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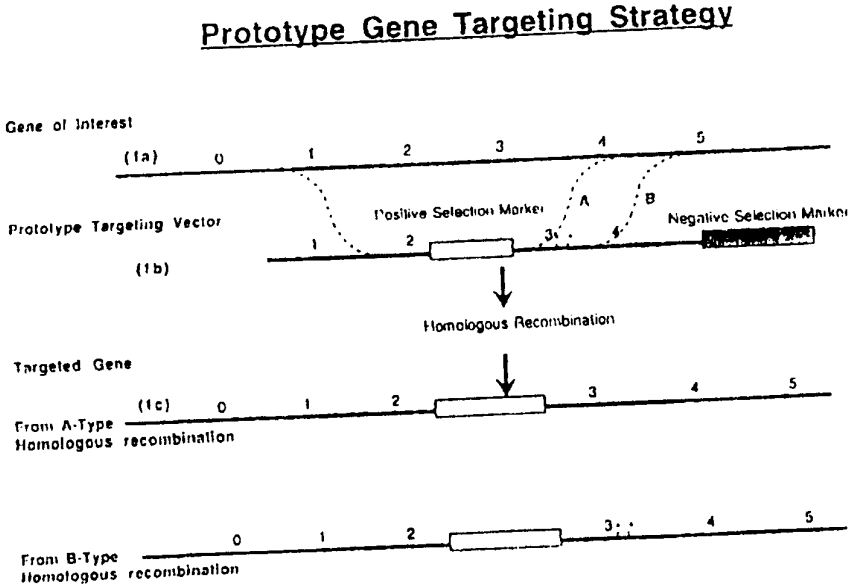

(57) Abstract

Disclosed is non-human mammal homozygous for a targeted amyloid precursor protein-encoding gene comprising: (1) a human Aβ peptide-encoding sequence in place of the native Aβ peptide-encoding sequence; and (2) Swedish FAD mutations. The non-human mammal may also lack one or both copies of the SOD-1 peptide-encoding sequence.

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GENE-TARGETED NON-HUMAN MAMMALS DEFICIENT IN SOD-1 GENE AND EXPRESSING HUMANIZED Aβ SEQUENCE WITH SWEDISH FAD MUTATION

Field of the Invention

This invention relates to gene-targeted non-human mammals and to animal models for human diseases.

Background of the Invention

Alzheimer's Disease (AD) is a human disease for which there is currently no effective treatment. AD is characterized by progressive impairments in memory, behavior, language, and visuo-spatial skills, typically progressing in severity over a 6 to 20-year period, ending in death.

The neocortex, amygdala and hippocampus of the brain are the primary sites of neuropathology in AD. The typical neuropathology of AD comprises extracellular neuritic plaques, intracellular neurofibrillary tangles, neuronal cell loss, gliosis and cerebral vessel amyloid deposition. The neuritic plaques consist of cores of amyloid protein fibrils surrounded by a rim of dystrophic neurites; the plaques have been suggested as the primary site of damage to the cortex. The major protein component of the amyloid protein of the plaque is known as the Aβ peptide, a 4 kD peptide comprising between 39 and 43 amino acids. The Aβ peptide that predominates in plaques has 40 or 42 amino acids.

The Aβ peptide is proteolytically derived from an integral membrane protein known as the β-amyloid precursor protein ("APP"). There are several APP isoforms (having 695, 751 or 770 amino acids), which are encoded by mRNA species resulting from alternative splicing of a common precursor RNA. The APP gene is encoded by a single copy gene found on human chromosome 21 (Estus et al., Science 255:726-728 (1992). The APP

gene product ("APP") is alternatively processed via two cellular pathways. Processing in the "amyloidogenic" pathway yields APP fragments bearing the Aβ peptide or the Aβ peptide itself. Alternatively, in the "nonamyloidogenic" pathway, APP is cleaved within the Aβ sequence. This results in destruction of the Aβ peptide and secretion of the large N-terminal ectodomain of APP. The Aβ peptide is produced and secreted by a wide variety of cell types in various animal species. It has been found in body fluids, including serum and cerebral spinal fluid.

Complementary DNAs encoding human APP, have been cloned and sequenced. *See, e.g.*, Kang et al., *Nature* 325: 733-736 (1987); Goldgaber et al., *Science* 235:877-880 (1987); Tanzi et al., *Nature* 331:528-530 (1988); and Robakis et al., *Proc. Natl. Acad.Sci. USA* 84:4190-4194 (1987). The cDNA for a mouse homolog of human APP has also been cloned and sequenced. Human and murine APP amino acid sequences have a high degree of homology (96.8%), indicating that the protein is conserved across mammalian species (Yamada et al., *Biochem. Biophys. Res. Commun. 149*: 665-671 (1987)). The mouse Aβ and human Aβ sequences differ at positions 5, 10 and 13 (i.e., positions 676, 681 and 684 of the complete APP770 sequence). The amino acid changes, from mouse to human Aβ, are: Gly to Arg (Aβ 5, APP 676); Phe to Tyr (Aβ 10, APP 681); and Arg to His (Aβ 13, APP 684).

A form of Alzheimer's disease known as "Swedish Familial Alzheimer's Disease" has been associated with two mutations known as the "Swedish FAD mutations." The Swedish FAD mutations are transversions (G to T and A to C) in codons 670 and 671 (APP 770 transcript), which are in exon 16 of the APP gene (Mullan, *Nature Genetics* 1:345-347 (1992)). The Swedish FAD mutations change lysine to asparagine and methionine to leucine at

positions 670 and 671, respectively, in the amyloid precursor protein. These amino acid changes are immediately adjacent to the amino terminus of the Aβ peptide.

The Swedish FAD mutations may act by altering the proteolytic processing of APP so that increased amounts of Aβ are released (Cai et al., *Science 259*:514-516 (1993)). In vitro studies have demonstrated that cells expressing APP with the Swedish FAD mutation produce 3 to 7-fold more Aβ than cells expressing APP without the mutation. Furthermore, it was shown that the methionine to leucine mutation at amino acid 671 (M671L) is principally responsible for the increase in Aβ production (Citron et al., *Nature*, 360: 672-674 (1992)). A mutagenesis study to examine substrate requirements of proteases that cleave APP at the amino-terminus of Aβ in human cells grown in tissue culture showed that most amino acid substitutions at position 671 strongly inhibit Aβ production. Except the methionine-to-leucine substitution, the only substitutions at position 671 that did not decrease Aβ production were changes to tyrosine and phenylalanine, both of which are large and hydrophobic residues. Another amino acid that shares these characteristics (but was not tested) is tryptophan. A small number of substitutions at position 670 had no effect on Aβ levels (Citron et al., *Neuron* 14: 661-670 (1995)).

Genetically engineered non-human mammals may serve as models for at least some aspects of AD. The genetic engineering of non-human mammals (or any other organism) may be carried out according to at least two fundamentally different approaches: (1) random insertion of an exogenous gene into a host organism, and (2) gene targeting. The term "transgenic" has sometimes been used in a broad sense, to indicate any organism into which an

exogenous piece of DNA has been incorporated. As used herein, however, the term "transgenic" is reserved for organisms (i.e., non-human mammals) comprising a piece of exogenous DNA that has been randomly inserted. A transgenic organism expresses the transgene in addition to all normally-expressed native genes (except the gene or genes in which the random insertion(s) may have taken place).

Transgenic non-human mammals comprising human APP DNA sequences, in addition to the native APP DNA sequences, are known. *See, e.g.*, Quon et al., (*Nature* 352: 239-241 (1991)); Higgins et al., (*Annals NY Acad Sci.* 695:224-227 (1994); Sandhu et al.,(*J. Biol. Chem.* 266:21331-21334 (1991); Kammesheid et al., (*Proc. Natl. Acad. Sci. USA* 89:10857-10861 (1992); Lamb et al., (*Nature Genet.* 5:22-30 (1993); Pearson et al., (*Proc. Natl. Acad. Sci. USA* 90:10578-10582 (1993); McConlogue et al., (McConlogue et al., *Neurobiol. Aging* 15, s12 (1994); Games et al., (*Nature* 373:523-527 (1995); and U.S. Patent No. 5,387,742.

In contrast, a gene-targeted organism has had a selected native DNA sequence or gene (i.e., targeted gene) partially or completely removed or replaced through a process known as homologous recombination. If the targeted gene is a single-copy gene and the organism is homozygous at that locus, the gene-targeted organism can no longer express the targeted native gene. The organism may or may not express a modified version of the targeted gene, depending on whether the targeted gene was mutated into a modified, but functional form, or mutated into a null allele, i.e., "knocked out." An attempt to produce, by gene targeting, mice homozygous for an APP null allele (and thus devoid of APP), has been published (Muller et al., *Cell* 79:755-765 (1994)). This attempt, wherein exon 2 of the APP gene was disrupted, resulted in mice

expressing a shortened form of APP, at 5 to 10-fold lower levels than the expression of normal APP in wild type mice.

The molecular reduction of oxygen to water during oxidative phosphorylation results inevitably in the production of superoxide radicals ("$O_2^{\bullet-}$") that are reactive oxygen species containing an unpaired electron orbital. Superoxides act as either reductants or oxidants and can form other reactive species including the hydroxyl radical ("$OH^{\bullet}$") through interaction with iron (Haber-Weiss reaction) and peroxynitrite by reaction with nitric oxide. Reactive oxygen species attack proteins, DNA, and membrane lipids, thereby disrupting cellular function and integrity.

The primary defenses against the superoxide radicals are the superoxide dismutase enzymes (SOD) that catalyze the dismutation of superoxide to hydrogen peroxide. Three forms of SOD are known to exist in mammals: cytoplasmic SOD (Cu/Zn SOD), mitochondrial SOD (Mn SOD), and extracellular Cu/Zn SOD (EC-SOD). In mammals, SOD-1 is the gene that encodes Cu/Zn SOD, SOD-2 is the gene that encodes Mn SOD, and SOD-3 is the gene that encodes EC-SOD.

Cu/Zn SOD is a homodimeric protein of 32 kD that is localized to the cytoplasm and, perhaps, peroxisomes. It is produced constitutively in all cell types and is the most abundant SOD. High to moderate levels of Cu/Zn SOD are found in erythrocytes, the liver, skeletal muscle, and the brain. Mn SOD is a tetrameric protein localized to mitochondria and is found at approximately 5 to 10% of the levels of Cu/Zn SOD in cells. EC-SOD is a tetrameric protein evolutionarily related to Cu/Zn SOD that is found at low levels in plasma.

SOD-1 has been isolated and cloned from many different organisms. The complete amino acid sequences

of Cu/Zn SOD from 11 different species have been compared. A high degree of homology is evident among those of vertebrate origin and the metal binding sites appear to be conserved in all the species. See, e.g., Bannister et al., *CRC Critical Reviews in Biochemistry*, 22:111-180 (1987).

The human Cu/Zn SOD has 153 amino acids per monomeric subunit and is encoded by a single-copy gene on chromosome 21. See, e.g., Tan et al., *J. Exp. Med.*, 137: 317-330 (1973). Human SOD-1 and cDNA have been cloned and sequenced. See, e.g., U.S. Patent No. 5,196,335 (Groner); and U.S. Patent No. 5,252,476 (Hallewell et al.). A full-length cDNA for murine Cu/Zn SOD has been isolated (Bewley, *Nucleic Acids Res.* 16:2728 (1988)) and the structure of the single-copy gene on chromosome 16 has been reported (Benedetto et al., *Gene* 99:191-195 (1991)).

Oxidative stress has been implicated in normal aging and many human pathological conditions (Gutteridge, *Free Rad. Res. Comms.*, 19:141-158 (1993); Halliwell and Gutteridge, *Methods in Enzymology* 186:1-75 (1990)). Some examples include stroke, head and spinal cord trauma, Alzheimer's disease, atherosclerosis, Parkinson's disease, and Huntington's disease. Major sources of free radical production, including ischemia/reperfusion, inflammation, and mitochondrial injury, are common features of many of these conditions. Diseases in which reduced SOD activity may play a role include, for example, amyotrophic lateral sclerosis (ALS), Parkinson's disease, Fanconi's anemia and aluminum toxicity.

A detrimental role for the superoxide radical in human disease is supported in animal models of disease processes using transgenic mice overexpressing Cu/Zn SOD. Chan et al. (*Acta Neurochirurgica. Suppl.* 51:245-247 (1990)) reported that cortical neurons isolated from

transgenic mice overexpressing Cu/Zn SOD two to threefold relative to normal Cu/Zn SOD levels are protected against glutamate neurotoxicity *in vitro*. Neuroprotection is also conferred in Cu/Zn SOD transgenic mice against focal cerebral ischemia (Kinouchi et al., *Proc. Natl. Acad. Sci. USA* 88:11158-11162 (1991)) and N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced toxicity that causes damage similar to that observed in Parkinson's disease (Przedborski et al., *J. Neurosci.* 12:1658-1667 (1992)).

Evidence for a direct role of Cu/Zn SOD in human disease is exemplified by the disease ALS. ALS is a progressive paralytic disorder caused by the degeneration of large motor neurons of the brain and spinal cord and is usually fatal within five years of onset of symptoms. Approximately 90% of ALS is "sporadic", i.e., no familial history of the disease. Enhanced oxidative damage and stress in sporadic ALS patients, as evidenced by increases in protein carbonyl content and complex I electron transport activity, was reported by Bowling et al. (*J. Neurochem.* 61: 2322-2325 (1993)). Approximately 10 % of ALS is inherited as an autosomal dominant trait and is termed familial ALS (FALS). Recently, in a subset of FALS cases, more than 20 different missense mutations were identified within SOD-1 that resulted in a 40 to 50% reduction in the Cu/Zn SOD activity measured in red blood cell lysates. *See*, Rosen et al., *Nature* 362:59-62 (1993); and Deng et al., *Science* 261:1047-1051 (1993).

The role of reduced Cu/Zn SOD activity in FALS is unclear, however, because transgenic mice overexpressing human Cu/Zn SOD bearing one of the FALS mutations develop progressive motor neuron loss similar to that observed in the human condition (Gurney et al., *Science* 264:1772-1775 (1994)). Ripps et al. (*Proc. Natl. Acad. Sci. USA* 92:689-693 (1995)) report that transgenic mice bearing a

mutation in the mouse SOD-1 gene that corresponds to one of the changes in human FALS gene have high expression of the altered gene in the central nervous system which is associated with an age-related rapidly progressive decline of motor function accompanied by degenerative changes of motorneurons within the spinal cord, brain stem, and neocortex. The tissues of these transgenic mice had normal levels of total SOD activity. This suggests that the mutations confer a gain-of-function on the Cu/Zn SOD protein that contributes to disease onset. One possibility is that reduced Cu/Zn SOD activity measured in the FALS patients is a co-factor in the disease (Gurney, *Science* 266:1587 (1994)).

To determine whether decreased SOD activity could contribute to motor neuron loss, Cu/Zn SOD was inhibited chronically with antisense oligonucleotides or diethyldithiocarbamate in spinal cord organotypic cultures derived from rats. Chronic inhibition of Cu/Zn SOD resulted in the apoptotic degeneration of spinal neurons, including motor neurons. Motor neuron toxicity could be entirely prevented by the antioxidant N-acetylcysteine (Rothstein et al., *Proc. Natl. Acad. Sci. USA* 91:4155-4159 (1994)). Similarly, Troy et al. (*Proc. Natl. Acad. Sci. USA*, 91: 6384-6387 (1994)) reported that inhibition of Cu/Zn SOD synthesis by antisense oligonucleotides in cultured PC12 cells (rat pheochromocytoma cells) results in apoptotic-like cell death in undifferentiated and nerve growth factor(NGF)-differentiated cultures. The authors suggest that free radical production caused by inhibition of Cu/Zn SOD is responsible for induction of the cell death pathway.

It has been proposed that SOD is essential for normal aerobic life. See, e.g., Olanow, *TINS* 16:439-444 (1993). For example, non-mammalian SOD deficient organisms have been established which exhibit highly

deleterious characteristics. *Escherichia coli* lacking SOD activity exhibit an oxygen-dependent auxotrophy for branched chain amino acids. These organisms are unable to grow aerobically on minimal media, and are highly sensitive to the free radical-producing agents paraquat and hydrogen peroxide (Carlioz et al., *EMBO J.* 5:623-630 (1986)). Cu/Zn SOD deficient yeast (*Saccharomyces cerevisiae*) are intolerant to atmospheric levels of oxygen and are auxotrophic for lysine and methionine (Chang et al., *J. Biol. Chem.* 266:4417-4424 (1991)). Null mutations for Cu/Zn SOD in *Drosophila melanogaster* cause toxic hypersensitivities to oxidative stress conditions and a significant reduction in the adult lifespan (Phillips et al., *Proc. Natl. Acad. Sci. USA* 86: 2761-2765 (1989)).

Recently, it has been demonstrated that ß-amyloid interacts with endothelial cells on blood vessels to produce an excess of superoxide radicals, resulting in alterations in endothelial structure and function (Thomas et al., Nature 380:168-171 (1996)). The superoxide radical can damage the vascular endothelium by either initiating an attack on cellular proteins and lipids or by scavenging endothelium-derived relaxing factor that would enhance vasoconstriction and reduce vasodilation. This suggests that a reduction in SOD activity would exacerbate ß-amyloid-induced vascular damage and a possible therapeutic benefit against ß-amyloid toxicity could be gained by treatment with a superoxide scavenger such as SOD. See, Stamler, *Nature* 380:108-111 (1996); see also, Bradbury, *Nature* 347:750 (1996).

Summary of the Invention

We have discovered that when a humanized APP-encoding gene, comprising the human Aß peptide encoding

sequence and the Swedish FAD mutations, is expressed in a gene-targeted non-human mammal, the human Aβ peptide is produced in the non-human mammal's brain. In non-human mammals homozygous for the targeted APP gene, the human Aβ peptide is produced in the absence of native Aβ peptide. In non-human mammals heterozygous for the targeted APP gene, the human Aβ is produced in the presence of reduced levels of native Aβ peptide (with the reduced level of native Aβ peptide being approximately 50% of that normally produced in wild-type control animals). We have also discovered that when a humanized APP gene, comprising the human Aβ peptide encoding sequence and the Swedish FAD mutations, is expressed in the brain of a gene-targeted non-human mammal, amyloidogenic cleavage at the β-secretase site of APP is enhanced. As a result of this enhanced cleavage, we expect enhanced production of the human Aβ peptide in the brains of the gene-targeted non-human mammals, as compared to production of the native Aβ peptide in the brains of wild-type control animals. In non-human mammals homozygous for the targeted APP gene, the amount of human Aβ peptide produced is approximately twice the amount of human Aβ peptide produced in non-human mammals heterozygous for the targeted APP gene.

Accordingly, in one embodiment, this invention features a non-human mammal homozygous for a targeted APP gene comprising: (1) a human Aβ peptide-encoding sequence in place of the native Aβ peptide-encoding sequence; and (2) at least one Swedish FAD mutation. In another embodiment, the invention features a non-human mammal heterozygous for a targeted APP gene comprising: (1) a human Aβ peptide-encoding sequence in place of the native Aβ peptide-encoding sequence; and (2) at least one Swedish FAD mutation.

The non-human mammals of this invention may be used as tools or models to elucidate the role of human Aβ in AD pathology and symptomatology.

We have also generated heterozygous SOD-1 null non-human mammals, exemplified by a gene-targeted mouse lacking one normal copy (allele) of SOD-1, thereby producing a reduced amount of Cu/Zn SOD. Additionally, we have generated homozygous SOD-1 null non-human mammals exemplified by a gene-targeted mouse lacking both normal copies (alleles) of SOD-1, thereby producing no measurable amount of Cu/Zn SOD protein.

We have successfully cross-bred gene-targeted mice comprising the human Aß peptide encoding sequence and the Swedish FAD mutation with gene-targeted mice lacking both copies of SOD-1 (i.e., homozygous SOD-1 null mice). Offspring from different litters derived from subsequent generations were cross-bred to produce mice which comprise: a human Aß peptide-encoding sequence in place of the native Aß peptide-encoding sequence; at least one Swedish FAD mutation; and which lack both copies of the SOD-1 gene.

Accordingly, in one embodiment, this invention features a non-human mammal homozygous for a targeted APP gene comprising: (1) a human Aß peptide-encoding sequence in place of the native Aß peptide-encoding sequence; (2) at least one Swedish FAD mutation; and (3) an absence of both copies of the murine SOD-1 encoding sequence. Because of the implicated role of Aß in producing an excess of superoxide radicals, lack of the superoxide radical scavenger SOD-1 should further exacerbate the deleterious impact occasioned by Aß secretion. As such, these animals may also be used as tools or models to elucidate the role of human Aß in AD pathology and symptomology. These non-human animals can also be compared with the non-human animals disclosed

herein which are capable of expressing SOD-1 to further understand the role that SOD-1 may play in the pathology and symptomology of AD.

The non-human mammals of this invention also may be used as assay systems to screen for *in vivo* inhibitors of amyloidogenic processing of APP in the non-human mammal's brain, non-brain tissues, or body fluids. Accordingly, the invention features a method for screening chemical compounds for their ability to inhibit *in vivo* processing of APP to yield the human Aβ peptide in the brain, in non-brain tissues, or in body fluids (e.g., blood and cerebral spinal fluid), said method comprising the steps of: (a) administering said chemical compounds to a non-human mammal which may lack one or both copies of the SOD-1 peptide-encoding sequence and which are homozygous or heterozygous for a targeted APP gene comprising: (1) a human Aβ peptide-encoding sequence in place of the native Aβ peptide-encoding sequence; (2) Swedish FAD mutations; and (b) measuring the relative amounts of amyloidogenic and nonamyloidogenic processing of amyloid precursor protein in brain tissue, non-brain tissue, or body fluids (or some combination thereof) of said non-human mammal, at an appropriate interval after administration of said chemical compounds.

As used herein, "APP" means amyloid precursor protein.

As used herein, "APP770" means the APP isoform that has 770 amino acid residues. The positions of the amino acid residues in the APP are numbered from 1 to 770, starting at the amino terminus.

As used herein, "arms of homology" means nucleotide DNA sequences in a targeting vector: (1) which have sufficient length and homology to provide for site-specific integration of part of the targeting vector

into the target gene by homologous recombination; (2) in which, or between which are located one or more mutations to be introduced into a target gene; and (3) which flank a positive selectable marker.

As used herein, "deletion vector" means a vector that includes one or more selectable marker sequences and two sequences of DNA homologous to the genomic DNA that flank the DNA gene sequence which is to be deleted.

As used herein, "homologous sequence" means a sequence at least about 90%, but preferably about 95%, identical to the corresponding target sequence. These flanking sequences are the arms of homology. Preferably, the arms of homology for the SOD-1 gene are substantially isogenic for the corresponding flanking sequences in the cell being targeted or "target cell."

A "substantially isogenic" sequence is at least about 97-98% identical to the corresponding target sequence. The use of DNA isogenic to the target cells helps assure high efficiency of recombination with the target sequences. The cumulative region of homology is longer than about 50 bp but is preferably about 2 kb or greater.

As used herein, "gene-targeted non-human mammal" means a non-human mammal comprising one or more targeted genes. The preferred gene-targeted non-human mammal is a mouse.

As used herein, "homologous recombination" means rearrangement of DNA segments, at a sequence-specific site (or sites), within or between DNA molecules, through base-pairing mechanisms.

As used herein, "humanized APP" means a non-human mammalian APP in which the native Aβ peptide sequence of the APP has been replaced with the human Aβ peptide sequence, and the remainder of the APP molecule, i.e., everything except the Aβ peptide sequence, is unchanged.

An APP is said to be "humanized" because it consists of a combination of human and native sequences.

As used herein, "human Aβ peptide" means a peptide having the amino acid sequence of the human Aβ peptide, regardless of whether the peptide is proteolytically derived from a human APP or a humanized APP. An Aβ peptide is said to be human, as opposed to humanized, because it consists exclusively of a human sequence.

As used herein, "normal" or "normal copy" in reference to SOD-1 or SOD-1 allele means the gene expressing wild type amounts of enzymatically active Cu/Zn SOD protein in a wild type mammal whose genome includes such SOD-1. Thus, an animal lacking at least one normal copy of an allele, as defined herein, need not necessarily have that allele excised form the genome of that animal; rather, the gene sequence can be sufficiently disrupted such that the expression of a protein encoded thereby is disrupted. Therefore, a mammal lacking at least one copy of a normal SOD-1 allele can, as defined herein, have a mutated SOD-1 allele that disrupts expression of the Cu/Zn SOD.

As used herein, "reduced amount" in reference to the amount of Cu/Zn SOD protein expressed in a gene-targeted mammal lacking one normal copy of SOD-1 gene means between about 25% and about 75% of wild-type Cu/Zn SOD protein typically expressed in a comparative mammal (e.g., a mouse in the case of the gene-targeted mouse).

As used herein, "no measurable amount" in reference to Cu/Zn SOD protein expressed in a gene-targeted mammal lacking both copies of normal SOD-1 means less than about 10% of wild-type Cu/ZN SOD protein normally expressed in a comparative mammal. Methodologies for measurement of protein expressed by a gene are varied and well-known; analyses may be made, for example, using anti-Cu/Zn SOD protein antibody

measurements of tissue samples such as exemplified in Example 14 below.

As used herein, "Swedish FAD mutations" means transversions (G to T and A to C) in codons 670 and 671 (APP 770 transcript), which are in exon 16 of the APP gene. The Swedish FAD mutations change lysine to asparagine and methionine to leucine at positions 670 and 671, respectively, in the amyloid precursor protein.

As used herein, "target gene" means a gene in a cell, which gene is to be modified by homologous recombination with a targeting vector.

As used herein, "targeted gene" means a gene in a cell, which gene has been modified by homologous recombination with a targeting vector.

As used herein, "targeting vector" means a DNA molecule that includes arms of homology, the nucleotide sequence (located within or between the arms of homology) to be incorporated into the target gene, and one or more selectable markers.

As used herein, "wild-type control animal" means a non-gene-targeted, non-human mammal of the same species as, and otherwise comparable to (e.g., similar age), a gene-targeted non-human mammal. A wild-type control animal can be used as the basis for comparison, in assessing results associated with a particular genotype.

As used herein, "about" in reference to a numerical value means "+/- 10%" of the numerical value, e.g., "about 10%" means between 9% and 11%.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are

described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Figures

Fig. 1 is a schematic diagram illustrating general principles of gene targeting.

Fig. 2 is a set of mouse APP genomic clone maps. Single letter abbreviations for restriction endonucleases are as follows: E, EcoRI; H, HindIII; X, XbaI

Fig. 3 is a diagram illustrating a FLASH™ restriction mapping method.

Fig. 4 is diagram illustrating the strategy for placing APP exons 15, 16 and 17 on the genomic APP restriction map.

Fig. 5 is a pair of genetic maps illustrating the relationship between APP exon 16 and the pAPP-TV replacement vector. Single letter abbreviations for restriction endonucleases are as follows: E, EcoRI; H, HindIII; X, XbaI; and N, NotI.

Fig. 6 is a schematic diagram illustrating the construction of plasmid pPNTlox^2.

Fig. 7 is a schematic diagram illustrating the construction of plasmids pAPP3'homol-4 and pAPP3'homol-7.

Fig. 8 is a schematic diagram illustrating the construction of plasmids pAPP5'homol-11 and pAPP5'homol-17.

Fig. 9 is a schematic diagram illustrating the restriction mapping of the 3′ arm of homology.

Fig. 10 is a pair of restriction maps for the APP 3′ and 5′ arms of homology.

Fig. 11 is a schematic diagram illustrating the construction of plasmid pAPP3′homolΔB.

Fig. 12 is a partial sequence of mouse APP exon 16, showing amino acid changes.

Fig. 13 is a schematic diagram illustrating the construction of plasmid pAPP3′homolΔB-NL.

Fig. 14 is a schematic diagram illustrating the construction of plasmid pAPP3′homolΔB-NLh.

Fig. 15 is a schematic diagram illustrating the construction of plasmid p4Z3′homolNL-h.

Fig. 16 is a schematic diagram illustrating the construction of plasmid pSK3′homolNL-h.

Fig. 17 is a schematic diagram illustrating the construction of plasmid pPNT3′homol.

Fig. 18 is a schematic diagram illustrating the construction of plasmid pAPP-TV.

Fig. 19 is a schematic diagram illustrating the strategy to detect homologous recombination within mouse APP. Single letter abbreviations for restriction endonucleases are as follows: E, EcoRI; H, HindIII; X, XbaI; and N, NotI.

Fig. 20 is a schematic diagram of APP, relevant carboxyl-terminal derivatives (CTD) and APP-specific antibodies.

Fig. 21 is a photograph of an immunoblot used to detect human Aβ epitopes from targeted ES cells.

Fig. 22 is a photograph of immunoblots used for detection of 12 kD and 9 kD carboxyl-terminal derivatives of APP in targeted mouse brain.

Fig. 23 is a photograph of immunoblots used for detection of 12 kD and 9 kD carboxyl-terminal derivatives of APP in targeted mouse brain.

Fig. 24A is a photograph of immunoblots used for the detection of human Aß in human and targeted mouse brains.

Fig. 24B is a photograph of immunoblots used for the detection of human Aß in human and targeted mouse brains.

Fig. 24C is a graph summarizing data on levels of human Aß in brains from the various genotypes of the APP gene-targeted mice

Fig. 25A is a set of mouse SOD-1 genomic clone maps.

Fig. 25B is a composite map based on the maps in Fig. 25A.

Fig. 26 is a schematic diagram of restriction mapping with the FLASH® Nonradioactive Gene Mapping Kit. A typical restriction map for a genomic clone isolated from a Lambda DASH® II library is shown at the top of the figure.

Fig. 27 is a SOD genomic map (upper portion) and a map of a targeting vector (lower portion).

Fig. 28 is a schematic diagram of the construction of intermediate plasmid pPNTlox^2.

Fig. 29 is a schematic diagram of the construction of intermediate plasmid pSK18-9.

Fig. 30 is a schematic diagram of the construction of intermediate plasmid pSOD3'homolTV.

Fig. 31 is a schematic diagram of the construction of intermediate plasmid pSK EH69-2.

Fig. 32 is a schematic diagram of the construction of deletion vector pSOD-TV.

Fig. 33 is a schematic diagram of the strategy used to detect homologous recombination within mouse SOD-

1. Fig. 34 is a graph summarizing data from the measurement of Cu/Zn SOD concentration and activity levels in blood samples from wild-type mice, and mice heterozygous and homozygous for the SOD-1 null allele.

Detailed Description

This invention provides a non-human mammal that produces the human Aβ peptide in its brain (and other tissues) instead of the Aβ peptide normally produced by that species of non-human mammal. A non-human mammal homozygous for a targeted APP gene produces the human Aβ peptide exclusively, i.e., it produces no native Aβ peptide. A non-human mammal heterozygous for a targeted APP gene produces both the human Aβ peptide and the native Aβ peptide.

The non-human mammal of this invention produces the human Aβ peptide exclusively by normal endogenous APP processing mechanisms. The APP undergoing such processing is advantageously expressed from genes having the normal copy number, and under the control of the endogenous APP expression control sequences. As a result, the APP in the non-human mammal of this invention is produced with the same developmental timing, same tissue specificity, and same rates of synthesis normally associated with native APP in the non-human mammal.

In the non-human mammal of this invention, the Aβ peptide produced is exclusively the human form, and it is produced at levels greater than the levels at which endogenous Aβ peptide is produced in control animals. The enhanced production of the Aβ peptide presumably results from the Swedish mutations exerting an effect on the normal APP processing mechanisms. Overexpression of, and increased pools of, APP are advantageously avoided.

APP has been shown to have a number of biological effects including inhibition of serine proteases,

(Oltersdorf et al., *Nature 341:* 144-147 1989)), cellular growth regulation (Saito et al., *Cell* 58: 615-622 (1989)), and cell attachment (Breen et al., *J. Neurosci. Res.* 28: 90-100 (1991)). In addition, APP and secreted forms of APP have been shown to be neuroprotective when overexpressed in transgenic mice, possibly through stabilization of intracellular calcium levels (Mucke et al., *Brain Res.* 666:151-167 (1994); Mattson et al., *Neuron* 10:243-254 (1993)). This is significant, because increased pools of APP may interfere with Aβ-specific pathogenic processes in unpredictable ways or initiate pathogenic events unrelated to the Aβ peptide or AD.

A further advantage of the present invention is that, in the non-human mammals homozygous for the targeted APP gene, the enhanced levels of human Aβ peptide are obtained *in vivo*, in the absence of native non-human Aβ peptides. This is significant, because the native Aβ peptide may have different properties than does the human Aβ peptide. *See, e.g.*, Otvos et al., *Eur. J. Biochem.* 211:249-257 (1993); and Bush et al., *Science* 265:1464-1467 (1994).

Because SOD is the initial defense against oxygen toxicity, and cytoplasmic Cu/Zn SOD represents a large fraction of SOD activity in mammals, it was not predictable whether mammals completely lacking Cu/Zn SOD, i.e., null for both alleles, could survive. Using methods for introducing gene-targeted mutations in mammals, currently exemplified in the art using mice, we wanted to determine whether ablation or "knock-out" of the mouse normal SOD-1 gene could be accomplished resulting in gene-targeted, non-human mammals deficient in or lacking cytoplasmic Cu/Zn SOD activity. We also wanted to determine whether mammals lacking Cu/Zn SOD activity would be viable. Such mutagenized mammals, when cross bred with the non-human animals disclosed above,

are useful for directly addressing the role of oxidative stress in any AD in which free radical damage has been implicated.

The gene-targeted non-human mammals of this invention may be used as tools or models to elucidate the role of human Aβ in AD pathology and symptomatology. The gene-targeted non-human mammals of this invention also may be used as assay systems to screen for *in vivo* inhibitors of amyloidogenic processing of APP to yield the human Aβ peptide in their brains, non-brain tissues, or body fluids (e.g., blood and cerebrospinal fluid).

The first step in producing a gene-targeted non-human mammal of this invention is to prepare a DNA targeting vector. The targeting vector is designed to replace, via homologous recombination, part of the endogenous APP gene sequence of a non-human mammal, so as to "humanize" the Aβ peptide-encoding part of the endogenous APP gene and introduce the Swedish mutations. The targeting vector is used to transfect a non-human mammalian cell, e.g., a pluripotent, murine embryo-derived stem ("ES") cell. In this cell, homologous recombination (i.e., the gene-targeting event) takes place between the targeting vector and the target gene. The mutant cell is then used to produce intact non-human mammals (e.g., by aggregation of murine ES cells to mouse embryos) to generate germ-line chimeras. The germline chimeras are used to produce siblings heterozygous for the mutated targeted gene. Finally, interbreeding of heterozygous siblings yields non-human mammals (e.g., mice) homozygous for the mutated targeted gene.

Targeting vectors for the practice of this invention can be constructed using materials, information and processes known in the art. A general description of the targeting vector used in this invention follows.

A targeting vector for use in this invention has two essential functions: (1) to integrate specifically (and stably) at the endogenous APP target gene; and (2) to replace a portion of exon 16 of the endogenous APP gene, thereby introducing the Swedish mutations and the mutations that convert the endogenous Aβ sequence to the human Aβ sequence. Those two essential functions depend on two basic structural features of the targeting vector.

The first basic structural feature of the targeting vector is a pair of regions, known as "arms of homology," which are homologous to selected regions of the endogenous APP gene or regions flanking the APP gene. This homology causes at least part of the targeting vector to integrate into the chromosome, replacing part (or all) the APP target gene, by homologous recombination.

Homologous recombination, in general, is the rearrangement of DNA segments, at a sequence-specific site (or sites), within or between DNA molecules, through base-pairing mechanisms. The present invention relates to a particular form of homologous recombination sometimes known as "gene targeting."

Currently, gene-targeting protocols utilized in the art are defined by the mouse; however, as the state of the gene-targeting art progresses to other mammals (i.e., rats, pigs, rabbits, non-human primates), the technique and methods disclosed below can rapidly be adapted thereto.

The second basic structural feature of the targeting vector consists of the actual mutation(s) to be introduced into the target gene. In the present invention, those mutations are nucleotide changes yielding the following amino acid changes: Gly to Arg (Aβ 5, APP 676); Phe to Tyr (Aβ 10, APP 681); Arg to His (Aβ 13, APP 684). The mutation(s) to be introduced into

the APP target gene must be located within or between the arms of homology.

Gene targeting, which affects the structure of a specific gene already in a cell, is to be distinguished from other forms of stable transformation, wherein integration of exogenous DNA for expression in a transformed cell is not site-specific, and thus does not predictably affect the structure of any particular gene already in the transformed cell. Furthermore, with the type of targeting vector preferred in the practice of this invention (e.g., the one described below), a reciprocal exchange of genomic DNA takes place (between the arms of homology and the target gene), and chromosomal insertion of the entire vector is advantageously avoided.

The examples below describe the actual construction of an APP gene targeting vector (and its use) to mutate the murine Aβ peptide-encoding sequence so that it encodes the human Aβ peptide, and simultaneously to introduce the Swedish FAD mutations into the murine APP gene. One of ordinary skill in the art will recognize that numerous other targeting vectors could be designed to introduce the same mutations, using the principles of homologous recombination. Gene-targeted non-human mammals produced using such other targeting vectors are within the scope of the present invention. A discussion of targeting vector constraints and considerations follows.

The length of the arms of homology that flank the replacement sequence can vary considerably without significant effect on the practice of the invention. The arms of homology must be of sufficient length for effective heteroduplex formation between one strand of the targeting vector and one strand of a transfected cell's chromosome, at the APP target gene locus.

Increasing the length of the arms of homology promotes heteroduplex formation and thus targeting efficiency. However, it will be appreciated that the incremental targeting efficiency accruing per additional homologous base pair eventually diminishes and is offset by practical difficulties in target vector construction, as arms of homology exceed several thousand base pairs. A preferred length for each arm of homology is 50 to 10,000 base pairs.

There is considerable latitude in the choice of which regions of the APP target gene, chromosomal regions flanking the APP target gene are represented in the targeting vector's arms of homology. The basic constraint is that the base pairs to be changed in the APP target gene must lie within or between the arms of homology. The arms of homology may lie within the APP target gene, but it is not necessary that they do so. They may flank the APP target gene.

Preferably, the targeting vector will comprise, between the arms of homology, a positive selection marker. The positive selection marker should be placed within an intron of the target gene, so that it will be spliced out of mRNA and avoid the expression of a target/marker fusion protein. More preferably, the targeting vector will comprise two selection markers: a positive selection marker, located between the arms of homology, and a negative selection marker, located outside the arms of homology. The negative selection marker is a means of identifying and eliminating clones in which the targeting vector has been integrated into the genome by random insertion instead of homologous recombination. Exemplary positive selection markers are neomycin phosphotransferase and hygromycin β phosphotransferase genes. Exemplary negative selection

markers are *Herpes simplex* thymidine kinase and diphtheria toxin genes.

To eliminate potential interference on expression of the target protein, the positive selection marker can be flanked by short *lox*P recombination sites isolated from phage P1 DNA. Recombination between the two *lox*P sites at the targeted gene locus can be induced by introduction of *cre* recombinase to the cells. This results in the elimination of the positive selection marker, leaving only one of the two short *lox*P sites. (See U.S. Patent No. 4,959,317). Excision of the positive selectable marker from intron 15 is correlated with enhanced expression from the APP-targeted gene and as a consequence greater Aß production. The enhancement of APP expression when the positive selectable marker is excised is most likely because the marker carries its own RNA processing signals that interfere with efficient and faithful APP transcription. Accordingly, animals containing the above-disclosed APP mutations but lacking the positive selectable marker are preferred for measuring human Aß and screening for inhibitors of amyloidogenic processing of APP.

In the specific SOD-1 deletion vector disclosed herein, the positive selection marker is neo^r, a gene that encodes resistance to the neomycin analog G418, and the negative selection marker is the herpes simplex virus thymidine kinase gene (HSV-TK), a gene that encodes susceptibility to ganciclovir. Upon successful gene-targeting and homologous recombination, the positive selection marker is incorporated into the genome in place of the gene to be deleted within the arms of homology, thereby making the gene-targeted cells resistant to G418, while the negative selection marker is excluded, thereby maintaining the cells' resistance to ganciclovir. Thus, to enrich for homologous recombinants, gene-targeted

cells are grown in culture medium containing G418 to select for the presence of the neo^r gene, and ganciclovir to select for the absence of the HSV-TK gene.

Fig. 1 illustrates general principles of gene targeting. In Fig. 1, mutations to be incorporated into the target gene are indicated by asterisks. In targeting vector (Fig. 1), the arms of homology are regions from 1 to 2 and from 3-4. The arms of homology are placed in the vector on either side of (i.e., flanking) a DNA sequence encoding a positive selection marker. A gene encoding susceptibility to an otherwise nontoxic drug (negative selection marker) is placed outside the region of homology. When the targeting vector is transfected into cells and integrated into the target gene, with crossovers occurring in the desired regions, the positive selection marker is inserted into the genome between regions 2 and 3 in this example (making the transformed cells resistant to the positive selection agent) while the negative selection marker is excluded. To enrich for the desired recombinants, transfected cells are grown in a culture medium containing the positive selection agent to select for the presence of the positive resistance marker and the negative selection agent, to select for the absence of the negative resistance marker.

Mutations in the arms of homology may or may not be incorporated into the target gene as a result of homologous recombination, depending on where the crossovers take place. For example, when hypothetical double crossover "A" occurs (Fig. 1), i.e., both crossovers on one side of the mutations, the mutations are not incorporated into the target gene. When hypothetical double crossover "B" occurs (Fig. 1), i.e., with the mutations between the crossovers, the mutations are incorporated into the target gene.

For a general description of gene targeting, see, e.g., *Nature 336*:348 (1988). One of ordinary skill in the art will recognize that while the examples below disclose our most preferred strategy and targeting vector for the development of a gene-targeted murine model system, various methods for producing gene-targeted murine, and non-murine, non-human mammals are known, and other strategies and targeting vectors will be readily apparent. Furthermore, as new methods become available, additional strategies and targeting vectors will be apparent, and may be preferred. Accordingly, the following examples are not intended as, and are not to be construed as, limiting with respect to the disclosure or the scope of the claims. Other non-murine, non-human mammals are within the scope of the present invention.

It should be recognized from the foregoing discussion that the practice of the present invention requires a DNA clone comprising at least that region of the APP gene that includes the nucleotides to be replaced. Such necessary DNA clones may be obtained by a variety of means. The nucleotide sequence of the human APP gene is known. *See, e.g.*, Kang et al. (*supra*); Goldgaber et al. (*supra*); Tanzi et al. (*supra*); and Robakis et al. (*supra*). The necessary DNA clones may be obtained, for example, by following the APP gene cloning methods set forth in the publications cited above. Alternatively, the published sequences can be used for the complete chemical synthesis of the desired DNA or the chemical synthesis of oligonucleotides that can be used as probes or PCR primers, as tools to obtain the necessary DNA by conventional techniques.

The specific procedure followed to generate SOD-1 knock out mice is detailed below. The following restriction enzymes, and their single letter codes, are

referred to in the examples which follow: EcoRI (E), HindIII (H), KpnI (K), EcoRV (R), SalI (S) and NotI (N).

In order that the invention described herein may be more fully understood, examples are provided below. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting the invention in any manner. Throughout these examples, molecular cloning reactions, and other standard recombinant DNA techniques, were carried out according to methods described in Maniatis et al., *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory (1982) or Sambrook et al., *Molecular Cloning - A Laboratory Manual, 2nd Ed.*, Cold Spring Harbor Press (1989), using commercially available enzymes, except where otherwise noted.

Examples

Example 1 - Cloning of Mouse APP exon 16 region.

The mouse APP genomic DNA was isolated from a phage library created from 129/Sv mouse DNA partially digested with Sau3A and cloned into the BamHI site of Lambda DASH™. Approximately 1.2 x 10^6 recombinant bacteriophage were screened for the presence of APP sequences by hybridization with a 300 base pair (bp), radiolabelled APP-specific DNA probe. This probe was generated by polymerase chain reaction (PCR) amplification using primers ST41 (SEQ ID NO:1) and ST42 (SEQ ID NO:2), which hybridize to the 5′ end of exon 15 and the 3′ end of exon 17, respectively, on a human APP cDNA clone (Fig. 2). The amplified fragment was separated from other components of the reaction by electrophoresis on a 1.0% agarose gel, and purified using GeneClean®II (Bio 101, Inc., La Jolla, CA). Purified probe DNA was radioactively labelled with ^{32}P-dCTP by the random primer method using a commercially available kit

(Multiprime DNA Labeling System™; Amersham Life Sciences, Arlington Heights, IL).

From this screen, seven clones were identified that hybridized to the APP probe (i.e., λAPP26, λAPP29, λAPP23, λAPP18, λAPP13, λAPP17, and λAPP32). These clones were purified by limiting dilution and plaque hybridization with the APP probe.

For each clone, DNA was prepared from bacteriophage particles purified on a CsCl gradient. Restriction maps were then generated for each of the cloned inserts using the FLASH™ Nonradioactive Gene Mapping Kit (Stratagene™ Inc., La Jolla, CA). The method is depicted schematically in Fig. 3. This method of restriction mapping involves completely digesting 10 μg of the phage DNA with NotI, which cleaves the vector at both ends of the cloned insert, leaving a T3 bacteriophage promoter attached to one end, and a T7 bacteriophage promoter attached to the other end. The NotI-digested DNA was then subjected to an EcoRI partial digest. The products of the partial digest were visualized by ethidium bromide staining, and transferred to a GeneScreen™ membrane (NEN Research Products, Boston, MA), by capillary transfer. The membrane-bound DNA was hybridized with an alkaline phosphatase-labelled oligonucleotide specific for the T3 promoter (supplied with FLASH™ kit). After hybridization, the membrane was washed and developed with a chemiluminescence-yielding substrate and exposed to X-ray film for approximately 60 minutes.

The oligonucleotide probes effectively label one end of the insert. By determining the positions of the bands on the X-ray film and calculating the DNA size for which they correspond, it was possible to determine the position of the EcoRI sites relative to the T3 end of the insert (Fig. 3). The first probe was then stripped from the membrane, and hybridization was repeated with a

T7-specific probe, to determine the positions of the EcoRI sites relative to the T7 end of the insert. This process was repeated using the enzymes HindIII and XbaI. By comparing the restriction enzyme maps of the different overlapping clones a composite map was assembled. Of the seven original clones isolated, five independent clones were identified (Fig. 2).

Exons 15, 16 and 17 were next located on our restriction map by hybridizing exon-specific probes to complete digests of each of the five different lamda genomic clones. For example, 3 μg of DNA from each of the 5 different clones was completely digested with EcoRI. The digested DNA was resolved on a 0.8% agarose gel, visualized with ethidium bromide staining and transferred to a GeneScreen™ membrane (NEN Research Products, Boston, MA) by capillary transfer. The membrane-bound DNA was then hybridized with a DNA probe that specifically hybridized to sequences from mouse APP exon 16. This probe was generated by PCR using oligonucleotides ST47 (SEQ ID NO:3) and ST48 (SEQ ID NO:4), which hybridize to the 5′ and 3′ ends of exon 16 respectively. After hybridization, the membrane was washed and exposed to X-ray film (Fig. 4). This experiment revealed that all clones contained a 600 bp fragment that hybridized to the exon 16 probe. In addition, clone λAPP13 hybridized to a 5.1 kb fragment while clones λAPP18, λAPP26 λAPP23 yielded a fragment, in addition to the 600 bp fragment, of sizes 900 bp, 1.7 kb, and 3.6 kb respectively. By combining this information with the restriction map data for each lambda clone, exon 16 was placed on our map so that the EcoRI site in exon 16 (Yamada, et al., *supra*) corresponds to the EcoRI site at position 12.8 on our composite map. A similar procedure was used to identify the positions of exons 15 and 17 on our composite map, using exon 15 and

exon 17-specific probes and utilizing the restriction enzymes XbaI and HindIII in addition to EcoRI. The exon 15-specific probe was generated using PCR primers ST45 (SEQ ID NO:5) and ST46 (SEQ ID NO:6). The exon 17-specific probe was generated using the primers ST49 (SEQ ID NO:7) and ST50 (SEQ ID NO:8). These last two 100 bp exons could only be localized to within the limits of a 4.4 kb fragment and a 1.9 kb fragment respectively (Fig. 2).

Example 2 - Construction of Targeting Vector pAPP-TV

A 4.5 kb HindIII fragment (position 6.5 - 11.0 on our summary map; Fig. 2) was chosen as a 5′ arm of homology, and a 5.6 kb HindIII fragment (positions 11.0-16.6 on our summary map; Fig. 2) was chosen as a 3′ arm of homology, which would contain the desired mutations. These fragments were isolated and cloned into pBlueScript™ SK+ (Stratagene, LaJolla, CA) and then subcloned into the plasmid pPNTlox^2 (described below) which contained a neo^r gene, an HSV-TK gene and linker sequences to produce a replacement vector (pAPP-TV; Fig. 5). The vector of the example can contain loxP sites surrounding the neo^r cassette, to allow for excision of the positive selection marker; see, e.g., Sauer U. S. Patent No. 4,959,317.

Intermediate Plasmid pPNTlox^2

The starting plasmid was pPNT (Tybulewicz, et al., *Cell* 65:1153-1163 (1991)); obtained from Dr. Richard Mulligan, MIT, Cambridge, MA). Two oligonucleotide linkers, one on each side of the neo^r cassette, were inserted into pPNT to create the intermediate, pPNTlox^2 (Fig. 6). A double-stranded 79 bp 5′ linker was created by annealing two single-stranded oligonucleotides that

overlap at their 3′ ends and then filling in the remaining single-stranded regions with the Klenow fragment of DNA polymerase I. The oligonucleotides PNT Not (SEQ ID NO:9) and PNT Xho (SEQ ID NO:10) (150 ng of each) were combined in a 30 μl reaction mixture containing 5 U of Klenow polymerase, Klenow polymerase buffer and 2mM dNTPs (dATP, dCTP, dGTP, and dTTP). After incubation for 1 hour at 37°C, 5 μl of this reaction mixture was simultaneously digested with NotI and XhoI. In addition, 200 ng of pPNT was digested with NotI and XhoI. The digested plasmid was purified, using a 0.8% agarose gel, and treated with calf intestinal phosphatase according to standard methods. A quantity (66 ng) of the double digested linker was ligated to the double digested and phosphatase-treated pPNT DNA. Following DNA transformation of competent WM1100 *E. coli* with the ligated DNA (Dower, *Nucleic Acids Res.* 16:6127-6145 (1988)), plasmid DNA was isolated from ampicillin-resistant bacteria and subjected to restriction analysis. The desired recombinant plasmids were identified as having acquired SalI, HpaI and NsiI sites (present in the linker) while still retaining the NotI and XhoI sites of the starting plasmid. One such recombinant plasmid with a 79 bp linker sequence was identified and designated pXN-4 (Fig. 6).

A similar approach was used to insert a 40 bp 3′ linker between the XbaI and BamHI sites of pXN-4. The oligonucleotides used to synthesize the linker were PNT Xba (SEQ ID NO:11) and PNT Bam (SEQ ID NO:12). In this case, pXN-4 and the double-stranded linker DNA were digested with XbaI and BamHI. The purified fragments were ligated and transfected into competent WM1100 bacteria. Plasmid DNA was digested with XbaI and BamHI, end-labelled with ^{32}P-dCTP and Klenow polymerase, and resolved on an 8% acrylamide gel. The gel was dried and

exposed to X-ray film. The desired recombinant clones were identified by a 40 bp band liberated by a XbaI-BamHI double digest. The resulting plasmid was designated pPNTlox^2 (Fig. 6). This construct includes the neo^r cassette flanked by the loxP sequences (*see*, Sauer, *supra*).

To confirm the sequences of the inserted linkers, a fragment containing both linkers was isolated from pPNTlox^2 using NotI and EcoRI and cloned into pBlueScript™ SK+, for sequencing purposes. Identity of the linkers was confirmed by direct nucleotide sequencing (using T3 and T7 sequencing primers (Stratagene, La Jolla, CA) and Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH).

Subcloning Arms of Homology

The HindIII fragment to serve as the 3' arm of homology was isolated from λAPP13 by digesting 30 μg of the phage DNA with HindIII, resolving the digested DNA on a 0.8% agarose gel, visualizing the DNA with ethidium bromide staining and then excising the 5.6 kb fragment from the gel. DNA was purified from the gel using GeneClean®II (Bio 101 Inc., La Jolla, CA). Simultaneously, 1 μg of pBlueScript™ SK+ (Stratagene, LaJolla, CA) was digested with HindIII and subsequently purified by the same procedure. Approximately 400 ng of the purified lamda DNA and 100 ng of the purified plasmid DNA were combined in a 10 μl ligation reaction, and competent WM1100 *E. coli* cells were transformed with the ligation products. Plasmid DNA from transformants was screened by restriction analysis, and plasmids, pAPP3'homol-7 and pAPP3'homol-4 (Fig. 7) were isolated.

These plasmids were analyzed after HindIII digestion to detect the 5.6 kb APP fragment. Since the insert could be in either of two orientations, plasmid DNA was further screened by XbaI digestion. Clone

pAPP3'homol-4 had the APP insert oriented with the 5' end closest to the T7 promoter. In pAPP3'homol-7, the 5' end was next to the T3 promoter (Fig. 7) .

The 5' arm of homology (a 4.5 kb HindIII fragment) was similarly subcloned from λAPP23 into pBlueScript™ SK+. The clone in which the 5' end of this arm of homology is juxtaposed to the T3 promoter was called pAPP5'homol-17, while the clone in which the 5' end of this arm of homology is adjacent to the T7 promoter was called pAPP5'homol-11 (Fig. 8).

Restriction Mapping of Arms of Homology

Further restriction enzyme mapping was performed on the 3' arm of homology. Plasmids pAPP3'homol-4 and pAPP3'homol-7 were digested with BamHI, and the resulting fragments were resolved on an agarose gel, stained with ethidium bromide and visualized. Since a BamHI site is in the pBlueScript™ SK+ plasmid, in the multiple cloning site, near the T3 promoter, it was possible to determine the position of the BamHI site in the 5.6 kb APP fragment by determining the fragment sizes in each of the two digested samples (Fig. 9).

Positions of restriction sites that occurred once or twice in the 5.6 kb APP fragment were determined by the above method. If more than two sites of a given enzyme were present it became necessary to determine the relative positions by double-digesting each of the two plasmids with the enzyme in question as well as an additional enzyme which cut at sites capable of resolving ambiguities. The list of additional enzymes used to characterize this region includes: AccI, ApaI, BamHI, BstXI, ClaI, EagI, EcoRV, HincII, HpaI, KpnI, NsiI, PstI, SacI, SalI, SmaI, SpeI, and XhoI. A summary of these data is in Fig. 10. The same procedures were used to create a restriction enzyme map for the 5' arm. (Fig. 10).

Fragment from 3′ Arm of Homology

For the PCR mutagenesis strategy described below, a fragment of the 3′ arm was isolated. One μg of pAPP3′homo1-4 was digested with BamHI to generate two fragments: a plasmid backbone carrying the first 3.0 kb of the 3′ arm of homology and a 2.6 kb fragment representing the 3′ half of the 3′ arm of homology. Both fragments were isolated by gel electrophoresis. The 2.6 kb fragment was stored for later use. The 6.0 kb fragment that contained plasmid backbone attached to 3.0 kb of the arm of homology was re-ligated upon itself in order to generate a plasmid carrying the first 3.0 kb of the 3′ arm. This plasmid was called pAPP3′homolΔB (Fig. 11).

Mutagenesis of 3′ Arm of Homology

A total of 6 base pair changes were introduced into exon 16, using a 2-step PCR strategy (*see*, Fig. 12). In the first step, the K670N/M671L mutation, an XbaI site, and the first base pair change of the humanizing mutations were introduced. This was accomplished by first linearizing pAPP3′homolΔB using the enzyme NaeI. Ten ng of the linearized DNA was then included in each of two PCR reactions. The first reaction contained the primers ST58 (SEQ ID NO:13) and T7 (Stratagene, La Jolla, CA). This generated a 1.4 kb band that encompassed the 5′ end of the 3′ arm of homology to the 5′ junction of exon 16. This fragment also included the K670N/M671L mutation and a novel XbaI site that resulted as part of the K670N/M671L change.

The second PCR reaction used the primers ST59 (SEQ ID NO:14) and T3 (Stratagene, La Jolla, CA). This generated a 1.6 kb fragment that encompassed all of exon 16 to the BamHI site located in the middle of the 3′ arm of homology. This fragment also included the K670N/M671L mutation and XbaI site, as well as the first base pair change necessary to humanize Aβ (Fig. 13).

The product of the first reaction was purified using Magic™ PCR Preps DNA Purification System (Promega, Madison, WI) and digested with HindIII and XbaI in order to liberate the restriction sites at its ends. Similarly the product of the second reaction was purified and digested with XbaI and BamHI.

These two fragments, as well as HindIII and BamHI digested pGEM-4Z (Promega Corp., Madison, WI), were ligated and transfected into HB101 competent *E. coli* cells. DNA from the transformants was isolated and analyzed. A recombinant plasmid in which the two PCR fragments had joined at their XbaI sites and inserted into the BamHI and HindIII sites of pGEM™-4Z was designated pAPP3'homolΔB-NL (Fig. 13).

Using a similar strategy, a final PCR step to introduce the remaining mutations into exon 16 was employed, in order to convert it to the human sequence. Plasmid pAPP3'homolΔB-NL was linearized with NarI. The linearized DNA was amplified by PCR, using primers ST61 (SEQ ID NO:15) and Sp6 (Promega, Madison, WI). The 1.6 kb purified DNA fragment was digested with XbaI and BamHI and ligated to the 4.4 kb XbaI, BamHI-digested p3'homoΔDB-NL DNA fragment which had been isolated by gel electrophoresis. The resulting plasmid was designated pAPP3'homolΔB-NLh (Fig. 14).

To confirm the sequence of the mutagenized exon 16, direct nucleotide sequencing was performed, using the primers ST47(SEQ ID NO:3); which hybridizes to the 5' end of exon 16 and ST62(SEQ ID NO:16), which hybridizes to the intron region immediately 3' to exon 16. The 2.6 kb BamHI fragment, which was the 3' half of the 3' arm of homology, and which had been stored, was introduced back into the mutagenized half of the arm of homology. For this, plasmid pAPP3'homolΔB-NLh was linearized with BamHI and the previously purified 2.6 kb BamHI fragment was

ligated to it. The 2.6 kb BamHI fragment could insert into the plasmid in either of two orientations. Proper orientation was determined by AccI digestion. The correctly oriented BamHI fragment yields a 3.8 kb fragment while the incorrect orientation yields a 4.6 kb fragment (Fig. 15). The proper recombinant plasmid was designated p4Z3'homolNL-h.

In order to introduce the necessary restriction sites at either end of the mutagenized arm of homology, the arm was next "shuttled" into the vector pBlueScript™ SK+. The plasmid p4Z3'homolNL-h was digested with HindIII, the resulting 5.6 kb band was isolated by gel electrophoresis and cloned into the HindIII site of pBlueScript SK+. The orientation of the insert was determined by double digesting plasmid DNA with the enzymes AccI and SacI. A recombinant plasmid was chosen in which the 5' end was adjacent to the T7 promoter. This plasmid is designated pSK3'homolNL-h (Fig. 16).

Assembling Targeting Vector pAPP-TV

The plasmid pPNTlox^2 was prepared to receive the 3' arm of homology by digestion with EcoRI and then filling in the 4 base overhang using Klenow polymerase. Following further digestion with KpnI, the plasmid was isolated by gel electrophoresis. The 3' arm of homology was prepared as a 5.6 kb EcoRV, KpnI fragment (also isolated by gel electrophoresis) and cloned into the purified and digested pPNTlox^2 DNA. The resulting plasmid was designated pPNT3'homol (Fig. 17).

The 5' arm of homology was inserted into pPNT3'homol to give the final plasmid pAPP TV (Fig. 18). The 5' arm of homology was liberated by digesting plasmid DNA with XhoI, followed by filling-in the overhang with Klenow polymerase and then digesting with NotI. Plasmid pPNT3'homol was prepared by double digesting with NotI

and HpaI. The two DNA fragments were ligated and transfected into competent WM1100 *E. coli* cells.

Example 3 - Mutagenesis of Mouse APP Gene in ES cells

The R1 line of ES cells derived from pigmented 129/Sv x 129/Sv-CP F1 hybrid mice (Nagy, et al., *Proc. Natl. Acad. Sci. USA* 90: 8424-8428 (1993)), was obtained from Dr. Janet Rossant, Dr. Andras Nagy, Reka Nagy, and Dr. Wanda Abramow-Newerly (Mt. Sinai Hospital, Toronto, Ontario, Canada). The cells were grown in ES cell medium consisting of Dulbecco's Modification of Eagle's Medium (with L-glutamine and 4.5 g/L glucose; Mediatech, Herndon, VA) supplemented with 20% fetal bovine serum ("FBS"; Hyclone Laboratories, Logan, Utah; cat. # A-1115; Lot # 11152154), 0.1 mM non-essential amino acids (Mediatech 25-025-L1), 2 mM L-glutamine (Mediatech 25-005-L1), 10^{-6} M mercaptoethanol (Gibco 21985-023) 1 mM sodium pyruvate (Mediatech 25-000-L1), 1x concentration of a penicillin (5000 IU/ml) streptomycin (5000 mcg/ml) solution (Mediatech 30-001-L1) and 1000 U/ml of leukemia inhibitory factor (Gibco BRL 13275-029). The cells were grown on tissue culture plastic plates that had been treated with a solution of 0.1% gelatin (Sigma G9391) (gelatinized plates).

The cultures were grown in 100 x 15 mm plastic petri plates and were passaged every 48 hours, or when the cells became about 80% confluent. For passage, the cells were washed with phosphate buffered saline (without Ca^{2+} and Mg^{2+}) ("PBS") and then treated with a trypsin/EDTA solution (.05% trypsin, 0.02% EDTA in PBS). After all of the cells were in suspension, the trypsin digestion was stopped by the addition of ES cell medium. The cells were collected by centrifugation and resuspended in 5 ml of ES cell medium. A 1 ml aliquot of

the cell suspension was used to start a new plate of the same size.

Transfection of ES Cells

Plasmid DNA (400 μg) from pAPP-TV was prepared for electroporation by digesting it with Not I in a 1 ml reaction volume. The DNA was then precipitated with ethanol, washed with 70% ethanol, and resuspended in 500 μl of sterile water.

The NotI-linearized pAPP-TV DNA was electroporated into ES cells using a Bio-Rad Gene Pulser System (Bio-Rad, Hercules, CA). In each of 10 electroporation cuvettes, 40 μg of DNA was electroporated into 2.5×10^6 cells suspended in ES cell medium. The electroporation conditions used (250V and 500 μF) typically result in time constants ranging from 6.0 to 6.1 seconds. After electroporation, the cells were incubated for 20 minutes at room temperature in the electroporation cuvettes. The electroporated cells were then pooled and distributed equally onto 10 gelatinized plates. After 24 hours, the medium was aspirated and fresh ES cell medium was added. The next day, the medium in nine plates was replaced with ES cell medium supplemented with 150 μg/mL of G418 (Gibco) and 0.2 μM ganciclovir (Syntex, Palo Alto, CA) while one plate received medium supplemented only with 150 μg/mL of G418. After an additional 8 days of incubation, individual ES cell colonies were picked off the plates and separately expanded in a well of 24 well plates as described by Wurst et al. (*Gene Targeting Vol. 126* (Joyner, ed.), IRL Press, Oxford Univ. Press, pp. 33-61 (1993)). Comparison of the number of colonies that grew on the plates supplemented with G418 and ganciclovir versus the number that grew with only G418 supplementation was used to determine the efficiency of negative selection.

Analyses of ES cell transformants

When the cell culture in each well of the 24-well plates became approximately 80% confluent, it was washed and the cells were dispersed with two drops of trypsin-EDTA. Trypsinization was stopped by the addition of 1 ml of ES cell medium. An aliquot (0.5 ml) of this suspension was transferred to each of two wells of separate 24-well plates. After the cells had grown to near confluence, one of the plates was used for cryopreservation of the cell line while the other was used as a source of DNA for each of the cloned cell lines.

For cryopreservation, the cells in a 24-well plate were chilled by placing the plate on ice. The medium was replaced with fresh ES cell medium supplemented with 10% DMSO and 25% FBS, and the plate was cooled at approximately 0.5°C per minute, by insulating the plate in a styrofoam box and placing it in a -70°C freezer.

To isolate the DNA from the clones on the other plate, the medium in each well was replaced with 500 µl of digestion buffer (100 mM Tris-HCl, pH8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg/ml proteinase K). After overnight incubation at 37°C, 500 µl of isopropanol was added to each well and the plate was agitated for 15 minutes on an orbital shaker. The supernatant fluid was aspirated and replaced with 500 µl of 70% ethanol and the plate was shaken for an additional 15 minutes. The DNA precipitate was collected from the well and dissolved in 50 µl of TE solution (10 mM Tris-HCl pH 7.5, 1 mM EDTA).

The primary analysis for mutagenesis of the mouse APP gene involved a Southern hybridization screen of XbaI digested ES cell DNA. The 600 bp probe for this analysis was isolated from λAPP26 DNA digested with HindIII (Fig. 19). For the Southern hybridization screen, an aliquot

(10 μl) of each ES cell line DNA was digested with ApaI, resolved on an 0.8% agarose gel, and transferred to a GeneScreen™ membrane. The probe was labelled with ^{32}P-dCTP by random priming and hybridized overnight to the membrane at 58°C (Church et al., *Proc. Natl. Acad. Sci. USA 81*:1991-1995 (1984)). An ES cell line in which the APP gene has undergone the desired homologous recombination yields a 9.8 kb and 5.5 kb fragment in this assay (Fig. 19). This because homologous recombination introduces a novel XbaI site into the region where the neo^r cassette is incorporated. The 9.8 kb band results from the normal cellular copy of APP, while the 5.5 kb band results from the APP copy in which the novel XbaII site produces a shorter fragment. In this screen 22 cell lines (out of 248) were identified as potentially containing successfully targeted genes.

All of the cell lines scored as putative homologous recombinants by the primary screen were then further screened using a 300 bp probe isolated by first recovering a 3.2 kb EcoRI-SalI fragment from λAPP 32 (Fig. 2) and then further isolating the 300 bp probe from an EcoRV digest of the EcoRI-SalI fragment. This probe was hybridized to ApaI digested ES cell DNA. In this case, the normal APP gene yielded a 17 kb ApaI fragment and the mutant APP gene an 8 kb fragment (Fig. 19). Of the 22 cell lines examined in this screen, nine were shown to have undergone homologous recombination at the 3′ end.

Cell lines that were identified as having undergone homologous recombination by both screens were considered to have undergone *bona fide* homologous recombination. Depending on where the crossover occurs in the 3′ arm of homology, the mutations, may or may not incorporate into the gene (Fig. 1). We therefore carried out Southern hybridizations to detect the novel

XbaI site associated with the K670N/M671L mutation. For this, we used a 1.0 kb EcoRI-PstI fragment from p3'homolΔB (Fig. 10) to probe XbaI digested DNA. An unaltered APP gene yields an 9.8 kb band. An APP gene in which homologous recombination has taken place, but in which the planned mutations were not incorporated, yields a 3.7 kb band, while the inclusion of the desired mutations results in a 2.0 kb band. Of the nine bona fide homologous recombinant cell lines examined, four had incorporated the novel XbaI site.

DNA sequence analysis confirmed that the four cell lines with the novel XbaI site had each of the desired mutations. The DNA primers ST47 and ST62 were used to PCR amplify exon 16 from the mutant cell lines. The PCR products were purified using Magic™ PCR Preps DNA Purification System (Promega, Madison, WI) and cloned into pGEM®T (Promega, Madison, WI) according to the vendor's instructions. Theoretically, one half of the clones produced by this method will contain a PCR-amplified fragment from the non-mutagenized copy of exon 16. Clones carrying a mutagenized exon 16 were identified by the presence of an XbaI site in the cloned insert. DNA sequence analysis was then carried out using T7 and Sp6 primers (Promega, Madison, WI) and Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH). All four cell lines (73, 89, 139, and 148) which carried the exon 16 XbaI site had all of the desired mutations.

The mutagenized APP gene described here was designated APP^{nNLh} (wild-type APP gene is APP^{+}). The four ES cell lines bearing one copy of APP^{nNLh} were designated APP73, APP89, APP139, and APP148. Three of these lines were thawed, propagated, and used to produce chimeric mice.

Example 4 - Production of APP Gene-Targeted Mice

APP mutant ES cells were used to make chimeric mice by aggregating the mutant ES cells to E2.5 embryos and transferring the aggregated embryos to pseudopregnant females (Wood, et al., *Nature* 365:87-89 (1993)). ES cells were prepared for aggregation by limited trypsinization to produce clumps that average 10-15 cells. E2.5 embryos were collected from superovulated CD-1 female mice by oviduct flushing, as described by Hogan et al. (*Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1986)). The zona pellucida was removed from the embryos using acidic Tyrode's solution (Sigma Chemical Co., St. Louis, MO). Aggregation wells were created by pressing a blunt metal instrument (a darning needle) into tissue culture plastic. Embryos were then placed in a well with a clump of approximately 10-15 ES cells in a small drop (approximately 20 μl) of M16 medium (Sigma Chem. Co., St. Louis, MO) under mineral oil. After an overnight incubation (37°C, 100% humidity, 5% CO_2 in air), the aggregate embryos were transferred to the uterine horns of a pseudopregnant female. Contribution of the ES cells to the offspring was scored by the appearance of pigmented coat color. Pigmented mice are termed chimeric founders. Germline contribution by the ES cells was scored by the appearance of pigmented offspring from a cross between the chimeric founders and CD-1 females.

Of three mutant APP ES cell lines used in embryo aggregations, one gave two germline chimeras (Table 1).

TABLE 1

Cell Line No.	Number of Embryo Aggregation	Number of Pups Born	Number of Chimeric Founders	Number of Germline Chimeras
APP73	143	12	2	0
APP139	79	15	8	2
APP148	140	19	8	0

The germline chimeras were used to establish lines of mice carrying APP^{nNLh}. The presence of the mutant APP allele in the pigmented offspring was determined by Southern analysis (as described above) with genomic DNA prepared from a tail sample. Mice heterozygous for APP^{nNLh} (APP^{nNLh}/APP^{+}) have 8.8 kb and 2.0 kb XbaI fragments that hybridize with the 1.0 kb EcoRI-PstI probe. Mice homozygous for the mutant APP allele (APP^{nNLh}/APP^{nNLh}) were established by crossing two heterozygous mutant APP mice and were identified as having only a 2.0 kb XbaI genomic DNA fragment that hybridized with the 1.0 kb EcoRI-PstI probe.

Example 5 - Excision of the *neo* cassette

To remove the neo gene from intron 15 of the APP-targeted ES cells, 10 μg of circular pBS185 plasmid DNA (Sauer et al. *New Biol.* 2:441-449 (1990)) encoding the *Cre* recombinase was electroporated into the APP139 ES cell line using conditions described in example 3 except that only 700 ES cells were in the electroporation cuvette. After 20 minutes recovery at room temperature all of these cells were plated onto a gelatinized plate and grown in ES cell medium in the absence of G418 selection. Medium was changed every 48 hours. After 8 days, individual colonies were picked into 24 well plates and expanded. DNA was prepared from the expanded cell lines as described (Wurst et al., *Gene Targeting*,

(Joyner, ed.), Oxford Univ. Press, Oxford, England, pp. 33-61 (1993)). Loss of the PGK/*neo* gene was detected by Southern hybridization using the 300 bp EcoRI-EcoRI fragment from p3'homolΔB to probe XbaI digested DNA (see Fig. 10). An unaltered APP gene yields a 9.8 kb band, an altered APP gene in which the APP mutations and the neo^r gene are incorporated yields a 2.0 kb band, and an APP gene in which the neo^r gene has been excised, but the APP mutations remain incorporated, yields a 7 kb band. A total of 149 clones were screened, and 5 excision clones (neo^-) were identified.

A chimeric founder mouse produced by embryo aggregation with one of these neo^- clones (APP139-34) exhibited germline transmission of the mutant APP allele (termed APP^{NLh}). From this founder, heterozygous (APP^{NLh}/APP^+) and homozygous (APP^{NLh}/APP^{NLh}) lines for the neo^- APP mutant allele were established.

Example 6 - Expression of Humanized APP in Targeted ES Cells To determine if APP-targeted ES cell lines express full length APP containing the humanizing mutations in the Aβ domain, immunoblot analysis was done. ES cell lines 139, 89, and 73, along with the parental R1 ES cell line, were individually cultured in ES cell medium. After cells reached 80% confluence, the medium was changed to ES cell medium lacking serum and the cells were maintained at 37°C for 4 hours. Medium was collected and the proteins were concentrated by precipitation with 10% trichloroacetic acid (TCA), resuspended in 1x SDS sample buffer (Laemmli, *Nature* 227:680-685 (1970)) and boiled for 5 minutes. Samples were electrophoresed on 6% SDS-polyacrylamide gels and electroblotted onto nitrocellulose (Towbin et al., *Proc. Natl. Acad. Sci USA* 76:4350 (1979)). Filters were blocked with 5% nonfat evaporated milk in Tris-buffered

saline (TBS) (150 mM NaCl, 20 mM Tris-HCl, pH 7.4), followed by incubation with either antibody 6E10 (Kim et al., *Neurosci. Res. Commun.* 7:113-122 (1990))(1:2000) or antibody 22C11 (Weidemann et al., Cell 57:115-126 (1989)) (1:300). Antibody 6E10 (Fig. 20) was raised against residues 1 to 17 of the human Aβ peptide and has been shown to recognize human, but not rodent APP (Buxbaum et al., *Biochem. Biophys. Res. Commun.* 197:639-645 (1993)). Antibody 22C11 (Fig. 20) was raised against a peptide consisting of residues 60 to 100 of APP and recognizes human and rodent APP equally. Filters were incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (1:2000) (BioRad), followed by detection with enhanced chemiluminescence (ECL, Amersham).

The immunoblot results (Fig. 21) show that parental R1 cells synthesize an APP species that reacts with antibody 22C11 but not the human-specific 6E10 antibody. In contrast, the APP-targeted ES cell lines 139, 89 and 73 express and secrete an APP species that is recognized by antibody 6E10. This indicates that these cell lines express an APP protein containing the humanizing mutations present within the first 17 amino acids of the Aβ domain.

Example 7 - Expression of Humanized APP in Mouse Brain

Heterozygous APP-targeted mice (APP^{nNLh}/APP^{+}) derived from ES cell line 139 were used to test for the expression of the humanized APP in their brain cells. A heterozygous (APP^{nNLh}/APP^{+}) APP-targeted mouse and a littermate control mouse (APP+/APP+) were sacrificed at 2 months of age, and their brains were removed. Neocortex (ctx), hippocampus (hp), and cerebellum (Cb) were dissected from each fresh brain and subsequently frozen on dry ice. Approximately 50 mg of each tissue and ~50 mg normal human frontal cortex, were each sonicated in

0.2 ml of 150 mM NaCl, 50 mM Tris-HCl (pH 8), 1% Triton X-100, 0.2 mM PMSF (phenylmethyl-sulfonylfluoride; Sigma, St. Louis, MO) using a microtip. Extracts were then centrifuged at 14,000 x g, for 15 minutes, to remove insoluble material. Supernatant fluids were removed and saved, and the protein concentration of each sample was determined. To prepare samples for electrophoresis, 200 μg of each extract were precipitated in 4 volumes of ice-cold methanol and resuspended to 2.5 μg per μl in 1x Laemmli SDS sample buffer and boiled for 5 minutes. Samples were electrophoresed on 6% SDS-polyacrylamide gels and transferred to nitrocellulose by electroblotting. Filters were incubated with either antibody 6E10 (1:2000), or antibody 22C11 followed by goat anti-mouse IgG (BioRad) conjugated to horseradish peroxidase (1:2000) and bands were visualized by ECL (Amersham).

Immunoreactive species that co-migrate with the APP signal from human brain tissue were detected in extracts from neocortex (ctx), hippocampus (hp), and cerebellum (Cb) from the heterozygous APP^{nNLh}/APP^{+} mouse. As expected, no signal was detected in the control APP+/APP+ brain samples (Fig. 22A). Immunoblot analysis of the same samples with antibodies 22C11 (Figs. 20 and 22B) that recognize both mouse and human APP showed no significant qualitative or quantitative differences in APP immunoreactivity between the APP+/APP+ and APP^{nNLh}/APP^{+} mouse brains. These results indicate that APP with a humanized Aβ domain is being produced in the targeted mice.

Example 8 - Proteolytic Processing of Humanized APP in Mouse Brain

We predicted that the $FAD_{K670N/M671L}$ mutations would result in enhanced expression of the human Aβ peptide.

To assess the effect of the FAD$^{K670N/M671L}$ mutations on APP processing in the mouse brain, accumulation of the 12 kD C-terminal fragment produced after cleavage at the β-secretase site was measured in control (APP+/APP+) and heterozygous (APP^{nNLh}/APP^{+}) mouse brain tissues. Brain homogenates were initially immunoprecipitated with antibody 97 (Fig. 20), specific for the last 30 amino acids of human and mouse APP, to concentrate all APP fragments bearing C-terminal epitopes.

One-half brain (~0.2 g) from each of a normal littermate control and a heterozygous targeted mouse were separately homogenized in 3 ml of buffer B (20 mM Tris-HCl (pH 7.4), 2 mM EGTA, 1 mM EDTA, 1 mM benzamidine (Sigma), 1 mM DTT, and 1 mM PMSF). Extracts were centrifuged at 100,000 x g for 1 hour to fractionate the membrane and soluble fractions. Pellets, consisting of the membrane fraction, were washed in 3 ml of buffer B and re-centrifuged at 100,000 x g. Resulting pellets were then sonicated in 3 ml of 1x RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 0.5% deoxycholate, 1 mM benzamidine, 0.05 mM leupeptin (Sigma), 0.02 mM pepstatin A (Sigma). The sonicated extracts were centrifuged at 100,000 x g for 1 hour. Supernatant fluids were cleared prior to immunoprecipitation by incubating them for 1 hour with 2 μl of normal rabbit serum and 50 μl pansorbin (CalBiochem). After centrifugation at 3500 rpm for 10 minutes, 10 μl of rabbit antibody 97 and 30 μl of pansorbin were added to each supernatant fluid. The samples were incubated overnight at 4°C. Rabbit antibody 97, specific to the last 30 amino acids of APP conserved between human and rodent APP, was used to immunoprecipitate membrane-bound forms of APP. Immunoprecipitates were then obtained by centrifugation at 3500 rpm for 10 minutes. Pellets were resuspended in

high salt 1x RIPA (1x RIPA containing 350 mM NaCl) and centrifuged at 8500 rpm for 5 minutes. After 2 additional washes of the pellet in high salt 1x RIPA, the pellets were washed in 1x TBS and resuspended in 1x Laemmli SDS sample buffer. Samples were heated in boiling water for 5 minutes and then electrophoresed on 16% Tris-tricine polyacrylamide gels (Novex) to resolve carboxyl-terminal 9 kD and 12 kD fragments of APP. The proteins in the gels were then transferred to PVDF membranes by electroblotting and analyzed using either antibody 6E10 (Fig. 23A) or 4G8 (anti-Aβ 17-24); (Fig. 23B) Wiesniewski et al., *Acta Neuropathol.* 78:22 (1989).

The 12 kD fragment in APP^{nNLh}/APP^{+} brain homogenates is detected by antibody 6E10, which is specific for an epitope unique to the human Aβ peptide (epitope = human Aβ peptide residues 1-17). This confirmed that the mouse β-secretase recognized and cleaved the humanized APP. The 12 kD fragment was not detected in the control brain homogenates, because 6E10 is human-specific.

For a more direct measure on the efficiency of β-secretase cleavage between native and mutagenized APP, the immunoblots were reacted with antibody 4G8 (epitope = Aβ residues 17-24) which recognizes the 12 kD C-terminal fragment from both human and mouse APP, as well as the nonamyloidogenic 9 kD fragment produced after cleavage at the α-secretase site. A comparison of the 12kD to 9kD ratios is useful as a relative measure of APP processing through the amyloidogenic and nonamyloidogenic pathways. As shown in Fig. 23B, there was a significant increase in the 12 kD to 9 kD ratio in the APP^{nNLh}/APP^{+} versus control brain homogenates. This indicated that the presence of the $FAD_{K670N/M671L}$ mutations (and possibly the humanizing Aβ mutations) significantly enhanced cleavage at the β-secretase site in the mouse brain. Thus, there was an

increased amount of 12 kD peptide comprising the human Aβ peptide in the brain tissues of the mice of this invention as a result of the cleavage of APP at amino acid 672 (resulting from the action of the β secretase enzyme).

In addition to confirming that the 12 kD peptide comprising the human Aβ peptide is present in brain tissues of mice homozygous for the targeted APP gene, we have determined that the 4 kD human Aβ peptide is also present in brain tissues of the mice heterozygous for the targeted APP gene. On the basis of gene dosage effects, in general, and on the basis of APP measurements performed on our APP gene-targeted mice, in particular, the mice homozygous for the targeted APP gene produce, in their brains, approximately twice as much human Aβ peptide as do the mice that are heterozygous for the targeted APP gene (see Example 9, *infra*).

Example 9 - Detection of Human Aβ in the APP Gene-Targeted Mouse Brain

We have examined whether enhanced cleavage at the β-secretase site was associated with an increase in human Aβ accumulation in the APP gene-targeted mouse brain. An immunoprecipitation and immunoblotting method was employed using two distinct Aβ-specific antibodies. One-half mouse brain (0.25g) or normal human frontal cortex (70 year-old male) was homogenized in 3 ml 6M guanidine, 50 mM Tris, pH 7.5 and subsequently centrifuged at 100,000 x g for 1 hour. The supernatants were dialyzed against two changes of PBS containing 1 mM benzamidine, 1 μM pepstatin A, 1 μM leupeptin, 1 μM E64, and 100 μM PMSF (all protease inhibitors from Sigma Chemical Co., St. Louis, MO) overnight at 4°C. Dialysates were immunoprecipitated with 20 μl of antibody 1153 (anti-Aβ 17-40) (Siman et al. in *Research Advances in Alzheimer's*

Disease and Related Disorders, (Iqbal et al., eds.), Wiley and Sons, Chichester, England, pp. 675-684 (1995)). Immunoprecipitates were eluted in SDS-Tricine gel loading buffer (50 mM Tris/HCl, pH 6.8, 4% SDS, 12% glycerol, 2% ß-mercaptoethanol) at 90°C, resolved by electrophoresis on 16% Tris-Tricine SDS-polyacrylamide gels and proteins were transferred to PVDF membrane (Stratagene Cloning Systems). Immunoreactive bands were detected using 1:2000 antibody 6E10, followed by goat anti-mouse IgG conjugated to horseradish peroxidase and ECL.

A 4kD polypeptide that co-migrated with synthetic human Aß was detected in the brains of APP^{nNLh}/APP^{+}, APP^{nNLh}/APP^{nNLh}, APP^{NLh}/APP^{+} and APP^{NLh}/APP^{NLh} mice (Figs. 24A and 24B). Due to the selectivity of Ab 6E10 for human Aß, no immunoreactivity was found in the wild-type APP^{+}/APP^{+} mouse brain. Levels of immunoreactivity in the APP gene-targeted brains also corresponded directly with gene dosage, providing further evidence on the identity of the immunoreactive species as human Aß. The calculated levels of human Aß in brains from the various genotypes of the APP gene-targeted mice appear in Fig. 24C. An increase of approximately 50% in human Aß is correlated with excision of the *neo* selectable marker in mice that are heterozygous (APP^{nNLh}/APP^{+} and APP^{NLh}/APP^{+}) or homozygous (APP^{nNLh}/APP^{nNLh} and APP^{NLh}/APP^{NLh}) for the mutant APP allele. This is likely to be caused by more efficient transcriptional readthrough at the targeted locus due to removal of the neo^{r} gene cassette with its RNA processing signals. In the APP^{NLh}/APP^{NLh} mouse brain, human Aß levels were approximately 9-fold greater than those found in normal aged human brain.

Example 10 - Cloning of Mouse SOD-1

The mouse SOD-1 genomic DNA was cloned from a phage library created from 129/Sv mouse DNA partially

digested with *Sau*3A and inserted into the *Bam*HI site of Lambda DASH®II (Reaume et al., *Science, in press*, 1995). Using standard molecular biology techniques (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982) approximately 1.2 x 10^6 recombinant bacteriophages were screened for the presence of SOD-1 sequences by hybridization with a 900 base pair (bp), radiolabelled SOD-1 intron-specific DNA probe. This 900 bp probe was generated by polymerase chain reaction (PCR) amplification (Mullis and Faloona, *Methods in Enzymolology* 155:335-350 (1987)) of mouse genomic DNA using primers EH100 and EH101, which hybridize to each end of the SOD-1 intron 4 (Fig. 25B). Primer EH100 had the following sequence: 5′ ACCGGAATTC CATATAAGGA TATATACA 3′ (SEQ ID NO:17). Primer EH101 had the following sequence: 5′ TAGCGAATTC AGGTTTGAAT GATCAAGT 3′ (SEQ ID NO:18). The approximate placement of the 5 SOD-1 exons is shown in Fig. 25B, as based on data from Bendetto et al. (*Gene*, 99:191-195 (1991)), and marked above the map with bold numbers. The corresponding introns are numbered below the map in bold italics.

The amplified fragment was separated from the other components of the reaction by electrophoresis on a 1.0% agarose gel, and purified using GeneClean®II (Bio 101, Inc., La Jolla, CA). Purified probe DNA was radioactively labelled with ^{32}P-dCTP by the random primer method using materials and methods supplied by the kit manufacturer (Multiprime DNA Labeling System; Amersham Life Sciences, Arlington Heights, IL).

From this screen, 9 clones were identified which hybridized to the SOD-1 intron probe: λSOD12, λSOD72, λSOD20I, λSOD18, λSOD69, λSOD20G, λSOD47, λSOD67, and λSOD65 (Fig. 25A). These clones were purified by

limiting dilution and plaque hybridization with the SOD-1 intron 4 probe (Maniatis et al., 1982, *supra*).

For each clone, DNA was prepared from bacteriophage particles first purified on a CsCl gradient (Maniatis et al., 1982, *supra*). Restriction maps were then generated for each of the cloned inserts using the FLASH® Nonradioactive Gene Mapping Kit (Stratagene® Inc., La Jolla, CA), as summarized in Fig. 26. This method of restriction enzyme mapping involves first completely digesting 10 μg of the phage DNA with the restriction enzyme *Not*I using standard restriction enzyme digest conditions (Maniatis et al., 1982, *supra*). *Not*I cuts all clones in the vector DNA at either end of the cloned insert, leaving a T3 bacteriophage promoter attached to one end of the insert and a T7 bacteriophage promoter attached to the other end. The *Not*I digested DNA was then partially digested with the enzyme *Eco*RI, as an example, using limiting amounts of enzyme (0.2 units/μg DNA), in an 84 μl reaction volume at 37°C. Aliquots (26 μl) were removed after 3 minutes, 12 minutes, and 40 minutes and the digest reaction was stopped by the addition of 1 μl of 0.5 M EDTA. DNA from all three time points was resolved on a 0.7% agarose gel, visualized by ethidium bromide staining, and then transferred to a GeneScreen Plus® membrane (NEN® Research Products, Boston, MA) by capillary transfer (Maniatis et al., 1982, *supra*). The membrane was hybridized with an alkaline phosphatase labelled oligonucleotide that was specific for the T3 promoter (supplied with the FLASH® kit) using reagents and methods supplied by the kit manufacturer. After hybridization, the membrane was washed and developed with a chemiluminescent-yielding substrate and then exposed to X-ray film in the dark for approximately 60 minutes.

The oligonucleotide probes effectively label one end of the insert. By determining the positions of the

bands on the X-ray film and calculating the DNA size to which they correspond, it was possible to determine the position of the *Eco*RI sites relative to the T3 end of the insert. These results were then complemented by stripping the probe off of the membrane, and rehybridizing with a T7-specific oligonucleotide in order to determine the positions of the *Eco*RI sites relative to the T7 end of the insert. This process was repeated using the enzymes *Hind*III and *Kpn*I.

The results of restriction mapping of the 9 different SOD genomic clones using the FLASH® Nonradioactive Gene Mapping Kit (Stratagene® Inc., La Jolla, CA) are depicted in Fig. 25A. Some clones were isolated multiple times and therefore have more than one name. By comparing the restriction enzyme maps of the different overlapping clones, a composite map was assembled (Fig. 25B). Of the nine original clones isolated, a total of six independent clones were identified.

Example 11 - Construction of Deletion Vector

Examination of the published report that describes the structure of the mouse SOD-1 gene (Bendetto, et al., *supra*), revealed that the entire coding sequence of the mouse SOD-1 gene is within a 7.2 kb *Eco*RI fragment of DNA (Fig. 2B). Based on available restriction site data and preferred sizes for arms of homology (Deng et al., *Mol. Cell. Biol.*, 12: 3365-3371, 1992; Zhang et al., *Mol. Cell. Biol.*, 14: 2402-2410, 1994), a 4.9 kb *Hind*III-*Eco*RI fragment was selected for the 5′-arm of homology and a 3.3 kb *Kpn*I-*Eco*RV fragment was selected for the 3′ arm (Fig. 27). A targeting vector was created by isolating these two fragments and placing them into a plasmid which contained a neo^r cassette (a neomycin phosphotransferase gene linked to a phosphoglycerate kinase promoter) as a

positive selection marker, a TK cassette (a Herpes simplex virus thymidine kinase gene linked to a phosphoglycerate kinase promoter) as a negative selection marker, and linker sequences, to produce the deletion vector pSOD-TV. The process is set forth in detail below.

Construction of Intermediate Plasmid pPNTlox^2

Vector pSOD-TV was created from a derivative of pPNT (Tybulewicz et al., *Cell*, 65: 1153-1163 (1991)); obtained from Dr. Richard Mulligan, MIT, Cambridge, MA) by first inserting two oligonucleotide linkers on each side of the neo^r cassette creating the intermediate plasmid pPNTlox^2 (Fig. 28). A double-stranded 79 base pair 5′ linker having *Sal*I, *Hpa*I, and *Nsi*I sites was created by annealing two single-stranded oligonucleotides that overlap at their 3′ ends and then filling in the remaining single-stranded regions with the Klenow fragment of DNA polymerase I. The oligonucleotides PNT Not (5′ GGAAAGAATG CGGCCGCTGT CGACGTTAAC ATGCATATAA CTTCGTAT; (SEQ ID NO:9)) and PNT Xho (5′ GCTCTCGAGA TAACTTCGTA TAGCATACAT TATACGAAGT TATATGC; (SEQ ID NO:10)) (150 ng of each) were combined in a 30 μl reaction mixture containing 5 U of Klenow polymerase, Klenow polymerase buffer, and 2mM dNTPs (dATP, dCTP, dGTP, and dTTP). After incubating for 1 hour at 37°C, a portion (5μl) of this reaction mixture was simultaneously digested with the restriction enzymes *Not*I and *Xho*I to liberate the restriction enzyme sites at each end of the linker. In addition, 200 ng of pPNT was digested with *Not*I and *Xho*I. The digested plasmid was resolved on a 0.8% agarose gel, purified from the gel, and treated with calf intestinal phosphatase according to standard methods (Maniatis et al., 1982, *supra*). A quantity (66 ng) of the double digested linker was ligated to the double digested and phosphatase-treated pPNT DNA (Maniatis et

al., 1982, *supra*). Following DNA transformation of competent WM1100 *E. coli* cells (Dower, *Nucleic Acids Res.* 16:6127-6145 (1988)), plasmid DNA was isolated from ampicillin-resistant bacteria (Holmes et al., *Anal. Biochem.* 114:193-197 (1981)) and analyzed by restriction enzyme analysis. The proper recombinant plasmids were identified as having acquired *Sal*I, *Hpa*I, and *Nsi*I sites while still retaining the *Not*I and *Xho*I sites of the starting plasmid. One such recombinant plasmid with a 79 bp linker sequence was identified and designated pXN-4 (Fig. 28).

A similar approach was used to insert a 40 bp 3' linker between the *Xba*I and *Bam*HI sites of pXN-4. The oligonucleotides used to synthesize the linker were PNT Xba (5' CGTTCTAGAA TAACTTCGTA TAATGTATGC TAT; (SEQ ID NO:11)) and PNT Bam (5' CGTGGATCCA TAACTTCGTA TAGCATACAT TAT; (SEQ ID NO:12)). Plasmid pXN-4 and the double-stranded linker DNA were digested with *Xba*I and *Bam*HI. The purified fragments were joined by DNA ligation and transformed into competent WM1100 *E. coli* bacteria. Plasmid DNA was digested with *Xba*I and *Bam*HI, end-labelled with ^{32}P-dCTP and Klenow polymerase, and resolved on an 8% acrylamide gel (Maniatis et al., 1982, *supra*). The gel was dried and exposed to X-ray film. Proper recombinant clones were identified by the presence of a 40 bp band liberated by the *Xba*I-*Bam*HI double digest. The resulting plasmid was called pPNTlox^2 (Fig. 28). This construct includes the neo^r flanked by the loxP sequences; see Sauer, *supra*.

To confirm the sequences of the inserted linkers, a fragment containing both linkers was isolated from pPNTlox^2, using *Not*I and *Eco*RI, and cloned into pBlueScript®SK+, a vector more amenable to nucleotide sequencing. Identity of the linkers was confirmed by direct nucleotide sequencing (Sanger, *Proc. Natl. Acad.*

Sci. USA 74:5463-5467 (1977)) using T3 and T7 sequencing primers (Stratagene® Inc., La Jolla, CA).

Construction of pSOD-TV Deletion Vector

The deletion vector pSOD-TV was assembled by inserting the selected 5′ and 3′ arms of homology appropriately into pPNTlox^2. Initially, both arms of homology were subcloned from the phage inserts into pBlueScript® SK+. The map of λSOD18 (Fig. 25A) shows restriction enzymes sites for *Kpn*I (K), *Eco*RI (E), *Hind*III (H), and *Sal*I (S). The T3 and T7 promoters at either end of the cloned inserts are indicated. The 3′ arm of homology was isolated from λSOD18 by digesting 10 μg of bacteriophage DNA with the enzymes *Kpn*I and *Sal*I, resolving the digested DNA on a 0.8% agarose gel, and purifying the excised 3.3 kb fragment with GeneClean® II (Bio 101 Inc., La Jolla, CA). The same digest and gel isolation procedure were performed in parallel with pBlueScript®SK+ DNA except that the purified band was 3.0 kb. Approximately 400 ng of the purified lambda DNA and 100 ng of the purified plasmid DNA were combined in a 10 μl ligation reaction. Following transformation of competent WM1100 *E. coli*, plasmid DNA was isolated from ampicillin-resistant bacteria and analyzed by restriction enzyme analysis to identify the resultant plasmid pSK18-9 (Fig. 29).

To clone the 3′ arm of homology into pPNTlox^2 , the arm was liberated from pSK18-9 by enzymatic restriction with *Kpn*I and *Eco*RV and purified by gel isolation. The plasmid pPNTlox^2 was digested with *Eco*RI and the resultant 4 base overhang was filled-in using Klenow polymerase (Maniatis et al., 1982, *supra*). Following further digestion with *Kpn*I, the pPNTlox^2 plasmid DNA was gel purified and ligated to the purified 3′ arm of homology. Following bacterial transformation, proper recombinants were identified by restriction enzyme

analyses. The resulting plasmid was designated pSOD3'homolTV (Fig. 30).

A similar approach was used to isolate, purify, and subclone the 5' arm of homology into pBlueScript® SK+. The map of λSOD69 (Fig. 25A) shows restriction enzyme sites for *Kpn*I (K), *Eco*RI (E), and *Hind*III (H). The T3 and T7 promoters at either end of the cloned insert are indicated. The 5' arm was isolated from λSOD69 by first digesting the bacteriophage DNA with *Hind*III, isolating an 11.8 kb DNA fragment by gel electrophoresis, and then partially digesting this DNA fragment with limited amounts of *Eco*RI (1U/μg) for 1 to 5 minutes. The reaction was stopped with 20 mM EDTA. A 4.9 kb DNA fragment was purified after agarose gel electrophoresis and cloned into the *Eco*RI and *Hind*III sites of pBlueScript® SK+ to generate pSK EH69-2 (Fig. 31).

To construct plasmid pSOD-TV, the 5' arm of homology was removed from pSKEH69-2 by first digesting with *Not*I, filling-in the overhang with Klenow polymerase, and then digesting with *Sal*I. In parallel, pSOD3'homolTV was first partially digested with *Hpa*I, then completely digested with *Sal*I. The final deletion vector pSOD-TV was constructed by ligation of the two gel-purified fragments (Fig. 32).

Example 12 - Deletion of SOD-1 Gene in ES cells

Cells: The R1 line of ES cells derived from 129/Sv x 129/Sv-CP F1 hybrid mice (pigmented) (Nagy, et al., *Proc. Natl. Acad. Sci. USA* 90:8424-8428 (1993)) was obtained from Dr. Janet Rossant, Dr. Andras Nagy, Reka Nagy, and Dr. Wanda Abramow-Newerly (Mt. Sinai Hospital, Toronto, Ontario, Canada). The cells were grown in ES cell medium consisting of Dulbecco's Modification of Eagle's Medium

(Eagle's medium containing L-glutamine and 4500 mg/L glucose; Mediatech Inc., Herndon, VA) supplemented with 20% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, Utah; cat. # A-1115; Lot # 11152154), 0.1 mM non-essential amino acids (Mediatech 25-025-L1), 2 mM L-glutamine (Mediatech 25-005-L1), 10^{-6} M β-mercaptoethanol (Gibco 21985-023), 1 mM sodium pyruvate (Mediatech 25-000-L1), 1x concentration of a penicillin (50 IU/ml) streptomycin (50 mcg/ml) solution (Mediatech 30-001-L1), and 1000 U/ml of leukemia inhibitory factor (Gibco BRL 13275-029). The cells were grown on tissue culture plastic that had been briefly treated with a solution of 0.1% gelatin (Sigma G9391), i.e., gelatinized plates.

The cultures were plated at 1 X 10^5 cells per ml in 100 mm X 15 mm plastic culture plates and passaged every 48 hours, or when the cells became about 80% confluent. For passage, the cells were first washed with phosphate buffered saline without Ca^{2+} and Mg^{2+}, hereinafter referred to as "PBS", and then treated with a trypsin/EDTA solution (.05% trypsin, .02% EDTA in PBS). After all of the cells were in suspension, the trypsin digestion was stopped by the addition of ES cell medium. The cells were collected by centrifugation, resuspended in 5 ml of ES cell medium, and a 1 ml aliquot of the cell suspension was used to start a new plate of the same size.

DNA Gene-Targeting of ES cells

Vector pSOD-TV DNA (400 μg) was prepared for electroporation by digesting it with Not I in a 1 ml reaction volume. The DNA was then precipitated by the addition of ethanol, washed with 70% ethanol, and resuspended in 500 μl of sterile water.

The *Not*I-linearized pSOD-TV DNA was electroporated into ES cells using a Bio-Rad Gene Pulser® System (Bio-Rad Laboratories, Hercules, CA) as follows. In each of

10 electroporation cuvettes, 40 μg of DNA was electroporated into 5 x 10^6 cells suspended in 0.8 ml ES cell medium. The electroporation conditions were 250 V and 500 μF which typically result in time constants ranging between 5.7 - 6.2 seconds. After electroporation the cells were incubated for 20 minutes at room temperature in the electroporation cuvettes. All the electroporated cells were then pooled and distributed approximately equally onto 20 gelatinized plates (100 mm X 15 mm).

After 24 hours, the plates were aspirated and fresh ES cell medium was added. The following day, the medium in 19 plates was replaced with ES cell medium supplemented with 150 μg/mL of G418 (Gibco) and 0.2 μM ganciclovir (Syntex, Palo Alto, CA). The medium in one plate was supplemented with 150 μg/mL of G418 alone. After an additional 6 days, resultant individual ES cell colonies were picked off of the plates and separately expanded in individual wells of 24 well plates as described by Wurst et al., *Gene Targeting Vol. 126* (A. L. Joyner, ed.), IRL Press, Oxford University Press, Oxford, England, pp. 33-61 (1993). A comparison of the number of colonies that grew on the plates supplemented with G418 and ganciclovir versus the number that grew on the plates supplemented G418 alone was used to determine the efficiency of negative selection, which was 3.2 fold.

Analyses of Gene-Targeted ES cells

When the cell culture in each well of the 24-well plates became approximately 80% confluent, the cells were washed with PBS and then dispersed with two drops of trypsin-EDTA. Trypsinization was stopped by the addition of 1 ml of ES cell medium. An aliquot (0.5 mL) of this suspension was transferred to each of two wells of separate 24-well plates. After the cells had grown to

near confluence, one of the plates was used for cryopreservation of the cell line while the other was used as a source of DNA for each of the cloned cell lines.

For cryopreservation, the cells in a 24-well plate were first chilled by placing the plate on ice. The medium was then replaced with fresh ES cell medium supplemented with 10% DMSO and 25% FBS. The plate was then cooled at approximately 0.5°C minute by insulating the plate in a styrofoam box and placing it in a -70°C freezer.

To isolate the DNA from the cloned cell lines on the other 24-well plate, the medium in each well was replaced with 500 μl of digestion buffer (100 mM Tris-HCl, pH8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg/ml proteinase K) and incubated overnight at 37°C. After overnight incubation, 500 μl of isopropanol was added to each well and the plate was agitated for 15 minutes on an orbital shaker. The supernatant fluid was aspirated and replaced with 500 μl of 70% ethanol and the plate was shaken for an additional 15 minutes. The DNA precipitate was picked out of the well and dissolved in 50 μl of TE solution (10 mM Tris-HCl pH 7.5, 1 mM EDTA).

The primary analysis for deletion of the SOD-1 gene involved a Southern hybridization screen of *Apa*I digested ES cell DNA. The probe for this analysis was derived from the 5′ end of the SOD gene outside of the 5′ arm of homology (Fig. 33). An aliquot (10 μl) of each DNA sample was digested with *Apa*I, resolved on a 0.8% agarose gel, and transferred to a GeneScreen Plus® membrane. The probe was prepared by first isolating the 1.3 kb *Eco*RI-*Hind*III fragment from λSOD69 (Fig. 25A). Subsequent *Alu*I digest of this fragment yielded the 600 base pair probe. The probe was labelled with ^{32}P-dCTP by random priming and hybridized overnight to the membrane

at 58°C (Church et al., *Proc. Natl. Acad. Sci. USA* 81:1991-1995 (1984)). An ES cell line in which the SOD-1 gene has been successfully deleted yields 9 kb and 10 kb *Apa*I fragments, in this assay (Fig. 33). The targeting event replaces all of the SOD-1 coding sequence and introns with the neo^r positive selection marker. A normal SOD-1 gene carries an ApaI site located approximately 1.0 kb downstream from the 5′ border of the region to be deleted. The neo^r marker which replaces the SOD-1 gene carries with it an *Apa*I site near its 5′ end. As a result, this assay yields a 10 kb *Apa*I fragment from the normal SOD-1 gene and a 9 kb *Apa*I fragment from the deleted SOD-1 gene.

All cell lines scored as putative homologous recombinants by the primary screen were then further screened using a 1.8 kb *Eco*RI probe (isolated from an λSOD69, *Eco*RI digest) on *Spe*I digested ES cell DNA. In this case, the normal SOD-1 gene yielded a 9 kb fragment and the mutant SOD-1 gene a 10 kb fragment (Fig. 33). From 80 cell lines (numbered 1-80) whose DNA was analyzed, five were identified as having undergone proper homologous recombination. Three of the five cell lines that were identified as having undergone proper homologous recombination by both screens were then thawed and their cell numbers expanded. Cells from the resulting cultures were used to make chimeric mice.

Example 13 - Establishment of SOD-1 Null Mice

SOD-1 gene-targeted ES cells were used to make chimeric mice by aggregating the ES cells to E2.5 embryos and transferring the aggregated embryos to pseudopregnant females. (Wood et al., *Nature*, 365:87-89 (1993)). ES cells were prepared for aggregation by limited trypsinization to produce clumps that averaged 10-15 cells. E2.5 embryos were collected from superovulated

CD-1 female mice (albino) by oviduct flushing as described by Hogan et al., *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1986). The zona pellucida was removed from the embryos using acidic Tyrode's solution (Sigma Chemical Co., St. Louis, MO). Aggregation wells were created by pressing a blunt metal instrument i.e., a darning needle into tissue culture plastic. Embryos were then placed in a well together with a clump of approximately 10-15 ES cells in a small drop (approximately 20 μl) of M16 medium (Sigma Chemical Co., St. Louis, MO) under mineral oil. After an overnight incubation (37°C, 100% humidity, 5% CO_2 in air), approximately 20 of the aggregated embryos were transferred to the uterine horns of each pseudopregnant female (Hogan et al., *supra*). Contribution of the ES cells to the offspring was scored by the appearance of pigmented coat color. Pigmented mice were termed chimeric founders. Germline contribution by the ES cells was scored by the appearance of pigmented offspring from a cross between the chimeric founders and CD-1 females.

Three of the five gene-targeted ES cell lines were used in embryo aggregations. One (line 42) gave five germline chimeras, another (line 58) gave four germline chimeras, and the third (line 66) gave none (see Table 2).

Table 2

Cell Line Number	Number of Embryo Aggregations	Number of Pups Born	Number of Chimeric Founders	Number of Germline Chimeras
42	138	31	18	5
58	130	24	14	4
66	141	33	3	0

The germline chimeras were then used to establish lines of SOD-1 deficient mice and mice lacking SOD-1. The presence of the gene-targeted SOD-1 allele in the pigmented offspring was determined using the Southern blot strategy described above with genomic DNA prepared from a tail sample (Hogan et al., *supra*). Heterozygous SOD-1 null mice have 9 kb and 10 kb *Apa*I fragments that hybridize with the 5′ *Alu*I SOD probe. Homozygous SOD-1 null mice were established by crossing 2 heterozygous SOD-1 null mice and were identified as having only a 9 kb *Apa*I genomic DNA fragment that hybridized with the 5′ *Alu*I SOD probe.

Example 14 - Cu/Zn SOD Protein Levels and Enzymatic Activity in SOD-1 Null Mice

To confirm that the targeted disruption of the SOD-1 locus results in a reduction of Cu/Zn SOD levels in the tissues of the resulting mammals, blood samples were collected from wild-type mice, and mice shown to be heterozygous and homozygous for the SOD-1 gene. The blood samples were analyzed for Cu/Zn SOD protein by immunoblot analysis. Red blood cell lysates were prepared by lysing the blood cells (approximately 75 ul) by several cycles of alternately freezing and thawing. The protein concentrations of the cell lysates were determined using the BCA method (Pierce, Rockville, IL). An aliquot (2 to 2.5 μg of protein) of each sample was

electrophoresed on a 4-20% polyacrylamide gel (Novex, San Diego, CA) using a Tris/glycine/SDS (25 mM Tris/ 192 mM glycine/ 0.1%SDS) buffer system.

The separated proteins were transferred to nitrocellulose filters by electroelution and the resulting filters were blocked by incubation in blotto solution -- 5% non-fat, dry milk in 25 mM Tris-buffered saline (1x TBS) -- for 30 minutes. The filters were then submersed in a primary antibody solution (1:10,000 dilution in blotto solution) and incubated for between 2 and 18 hours. The primary antibody used was polyclonal rabbit antisera raised against purified mouse Cu/Zn SOD protein produced in *E. coli* (Hazelton Research Products, Denver, PA). The filters were washed three times for 5 minutes each in 1x TBS and incubated in secondary antibody solution (1:2,000 dilution in blotto solution) for two hours. The secondary antibody was a goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad, Richmond, CA). The filters were washed three times for 5 minutes each in 1x TBS and stained for alkaline phosphatase activity by incubating them for between 5 and 60 minutes in a commercially available alkaline phosphatase detection reagent (Bio-Rad, Richmond, CA).

Stained bands corresponding to Cu/Zn SOD protein were quantitated using a DocuGel V image analysis system and RFLPscan software (Scanalytics, Billerica, MA). The levels of Cu/Zn SOD protein are depicted in Fig. 34 (solid bars) and are expressed relative to the level of Cu/Zn SOD protein in the samples from wild-type mammals. The results of these studies indicate that the heterozygous SOD-1 null ($SOD1^-/SOD1^+$) mice exhibit an expected near 50% reduction in Cu/Zn SOD protein. Further, the homozygous SOD-1 null ($SOD1^-/SOD1^-$) mice showed no detectable Cu/Zn SOD protein on Western blots.

Cu/Zn SOD enzymatic activity in the red blood cell lysates was measured using the NADPH oxidation method of Paoletti, et al. (*Anal. Biochem.* 154:536-541 (1986)). Protein samples (1 mg) from the wild-type, heterozygous SOD-1 null mice, and homozygous SOD-1 null mice were extracted with an equal volume of ethanol:chloroform (2:1) and the resulting supernatant fluid was dialyzed overnight against PBS saline at 4°C. The protein concentration of the dialysate was determined using the BCA method (Pierce, Rockville, IL) and 10 ug of each sample was assayed for SOD activity. Enzymatic activity was expressed relative to the wild-type control sample (Fig. 34, cross-hatched bars). The Cu/Zn SOD activity of the sample from heterozygous SOD-1 null mammals was approximately 50% of that displayed by samples from the wild-type mammals, consistent with the expectation that only one SOD-1 allele was active in the heterozygous mammals. Furthermore, the protein sample derived from the homozygous SOD-1 null mice showed nearly a total reduction in Cu/Zn SOD activity. The small residual activity detected in this assay likely represents background activity associated with the assay, but could also reflect an endogenous superoxide scavenging activity supplied by an alternate protein.

Example 15 - Maintenance Conditions of SOD-1 Null Mice

Young adult heterozygous and homozygous SOD-1 null mice are currently being maintained under viral and antigen free conditions as defined by Charles River Laboratories, Wilmington, MA. Presently, the diet for these mice is the same as that provided to laboratory mice. No other unique or distinguishing living conditions have thus far been required for the mice.

Attempts at the breeding of homozygote males with homozygote females have been unsuccessful thus far, which

we believe is due to the deficiency of Cu/Zn SOD and the oxidative stress occasioned by pregnancy. The homozygote breeding pairs are capable of conceiving offspring, but the litters have been small in number (1-2) and the pups are either still-born, or those pups which are born alive die almost immediately.

The colony can be propagated by the breeding of heterozygous SOD-1 null males and females, homozygous SOD-1 null males to either wild type or heterozygous SOD-1 null females, or wild type males to homozygous SOD-1 null females.

Example 16 - Gene-Targeted Non-Human Mammals Deficient in SOD-1 Gene and Having Humanized Aß Sequence with Swedish FAD Mutation

To generate animals that lack Cu/Zn SOD and express only human Aß, a cross-breeding regimen was followed using the SOD-deficient and APP gene-targeted mice. This breeding strategy was complicated by the fact that both the SOD-1 and APP genes are found on chromosome 16 in the mouse and are in relative close proximity to one another. Initially SOD^{-}/SOD^{-} and APP^{NLh}/APP^{NLh} mice were crossed to produce offspring that contain a mutant SOD allele on one copy of chromosome 16 and a mutant APP allele on the other copy of chromosome 16 (*trans*-heterozygotes). Male progeny from this cross were then mated with CD-1 female mice in order to generate a population of animals in which a meiotic recombination event linking the SOD-1 and APP mutations on one copy of chromosome 16 could be identified (*cis*-heterozygotes). This identification was done on genomic DNA samples from portions of tail using a PCR method capable of distinguishing the mutant alleles from wildtype alleles for the SOD-1 and APP gene loci.

To identify the SOD-1 mutant locus, forward and reverse oligonucleotide primers were designed to amplify

a 417 bp segment of the PGK/*neo* gene that replaced the SOD-1 coding sequences in the SOD-deficient mice. The forward primer, designated neo28f, had the following sequence: 5′ GGATTGCACG CAGGTTCTCC 3′ (SEQ ID NO:19). The reverse primer, designated neo445r, had the following sequence: 5′ CCGGCTTCCA TCCGAGTACG 3′ (SEQ ID NO:20). This amplified product is found in PCR reactions with genomic DNA samples from SOD-1 mutant mice that are missing one copy of the SOD-1 gene ($SOD1^-/SOD1^+$), or both copies of the SOD-1 gene ($SOD1^-/SOD1^-$). It is absent, however, in PCR reactions with genomic DNA samples from mice with two normal copies of the SOD-1 gene ($SOD1^+/SOD1^+$).

To distinguish mice that have lost one or both copies of the SOD-1 gene, a second PCR method was employed using forward and reverse oligonucleotide primers designed to amplify a segment of the mouse SOD-1 gene spanning exon 4. The forward primer, designated EH128f, had the following sequence: 5′ ATCCACCTGA TGCTGTTTTA 3′ (SEQ ID NO:21). The reverse primer, designated EH129r, had the following sequence: 5′ CCAATGATGG AATGCTCTCC 3′ (SEQ ID NO:22). This 133 bp amplified product is found in PCR reactions with genomic DNA samples from mice with one or two normal copies of the SOD-1 gene ($SOD1^+/SOD1^+$ and $SOD1^-/SOD1^+$), but it is absent in PCR reactions with genomic DNA samples from mice lacking both SOD-1 gene copies ($SOD1^-/SOD1^-$).

The PCR reactions were done with 2 to 5 ug of genomic DNA using 2.5 units of Taq polymerase (Fisher) in a PCR buffer supplied by the vendor containing 1.5 mM $MgCl_2$. The PCR reaction consisted of 30 cycles of a 1 minute denaturation step at 95°C, a 1 minute annealing step at 55°C and a 1 minute extension step at 72°C.

To identify the APP gene-targeted locus from the APP^{NLh}/APP^{NLh} mice, forward and reverse oligonucleotide

primers were synthesized that span the *loxP* sequence found in intron 15 of the targeted APP locus that remained following excision of the PGK/*neo* gene by *cre*-mediated recombination. The forward primer, designated Hum1f, had the following sequence: 5′ CCTGGGTTGT AGGGACTGTA CTTG 3′ (SEQ ID NO:23). The reverse primer, designated Hum4r, had the following sequence: 5′ CACACCAAGA AGTACAATAG AGGG 3′ (SEQ ID NO:24). A 214 bp amplified product is obtained with genomic DNA having a normal mouse APP allele while a 298 bp fragment is obtained in a PCR reaction with genomic DNA having an APP gene-targeted allele. The PCR reaction conditions were identical to those described above except the annealing temperature was 60°C.

By using these PCR strategies in conjunction with one another, all potential genotypes resulting from the breeding regimen described above with the $SOD1^{-}/SOD^{-}$ and APP^{NLh}/APP^{NLh} mice can be identified and are summarized in Table 3.

TABLE 3

GENOTYPE	PCR PRODUCTS neo28f + neo445r PGKneo-specific	EH128f + EH129r SOD1-specific	Hum1f + Hum4r APP(intron 15)-specific
Wiltype	absent	133 bp	214 bp
cis-Heterozygotes	417 bp	133 bp	214 + 298 bp
Double Homozygotes[1]	417 bp	absent	298 bp

[1]The double homozygote genotype is $SOD1^{-}/SOD1^{-}$; APP^{NLh}/APP^{NLh}.

A screen of 138 progeny from the cross of male *trans*-heterozygotes with CD-1 females identified 6 *cis*-heterozygotes: three males and three females. Therefore, a sibling breeding scheme was carried out to

produce a colony of double homozygotes lacking Cu/Zn SOD and expressing only human Aß.

Double homozygotes lacking Cu/Zn SOD and expressing only human Aß were viable. They have manifested normal behavior and normal external gross anatomy up to 3 months of age. During those 3 months, housing and dietary protocols for the double homozygotes have been conventional, with no special requirements apparent.

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Cephalon, Inc.

(ii) TITLE OF INVENTION: GENE-TARGETED NON-HUMAN MAMMALS DEFICIENT IN SOD-1 GENE AND EXPRESSING HUMANIZED Aβ SEQUENCE WITH SWEDISH FAD MUTATIONS

(iii) NUMBER OF SEQUENCES: 24

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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(A) APPLICATION NUMBER:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATTGGATCCT TGAGCCTGTT GATGCCCGC 29

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATTAAGCTTC TCCACCACAC CATGATGAAT 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATTGGATCCG TTCTGGGCTG ACAAACA 27

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATTAAGCCTC AGTTTTTGAT GGCGGAC 27

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATTGGATCCT CGAGCCTGTT GACGCCCG 28

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATTAAGCCTC TGGTCGAGTG GTCAGAG 27

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATTGGATCCG TGTTCTTTGC TGAAGAT 27

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATTAAGCCTC TCCACCACGC CATGATG 27

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGAAAGAATG CGGCCGCTGT CGACGTTAAC ATGCATATAA CTTCGTAT 48

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCTCTCGAGA TAACTTCGTA TAGCATACAT TATACGAAGT TATATGC 47

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGTTCTAGAA TAACTTCGTA TAATGTATGC TAT 33

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGTGGATCCA TAACTTCGTA TAGCATACAT TAT 33

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AATTCTGCAT CTAGATTCAC TTCCGAGATC TCTTCC 36

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGCTCTAGAT GCAGAATTCA GACATGATTC AGGATTTGA 39

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GAATCTAGAT GCAGAATTCA GACATGATTC AGGATATGAA GTCCACCATC AAAAACTG 58

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CAATCTCGGG GAGAGGCAGT 20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACCGGAATTC CATATAAGGA TATATACA 28

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TAGCGAATTC AGGTTTGAAT GATCAAGT 28

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGATTGCACG CAGGTTCTCC 20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CCGGCTTCCA TCCGAGTACG 20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATCCACCTGA TGCTGTTTTA 20

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CCAATGATGG AATGCTCTCC 20

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCTGGGTTGT AGGGACTGTA CTTG 24

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CACACCAAGA AGTACAATAG AGGG 24

What is claimed is:

1. A mouse homozygous for a targeted amyloid precursor protein-encoding gene comprising: (1) a human Aβ peptide-encoding sequence in place of the native Aβ peptide-encoding sequence; and (2) at least one Swedish FAD mutation.

2. The mouse of claim 1, wherein said human Aβ peptide-encoding sequence encodes an arginine residue at Aβ position 5 (APP770 position 676), a tyrosine residue at Aβ position 10 (APP770 position 681), and a histidine residue at Aβ position 13 (APP770 position 684).

3. The mouse of claim 1, wherein said amyloid precursor protein-encoding gene encodes a leucine residue at APP770 position 671.

4. The mouse of claim 1, wherein said amyloid precursor protein-encoding gene encodes an asparagine residue at APP770 position 670, and a leucine residue at APP770 position 671.

5. The mouse of claim 1, wherein said human Aβ peptide is produced by endogenous mechanisms for processing amyloid precursor proteins.

6. The mouse of claim 5, wherein no murine Aβ peptide is produced.

7. The mouse of claim 5, wherein the rate of amyloid precursor protein synthesis in any given tissue is substantially the same as the rate of amyloid precursor protein synthesis in the corresponding tissue of a wild-type control mouse.

8. The mouse of claim 5, wherein production of said human Aβ peptide in any given tissue is greater than the production of native Aβ peptide in the corresponding tissue of a wild-type control mouse.

9. A mouse homozygous for a targeted amyloid precursor protein-encoding gene comprising: (1) a human Aβ peptide-encoding sequence in place of the endogenous murine Aß peptide-encoding sequence; and (2) a sequence encoding, at the APP position corresponding to murine APP770 position 671, an amino acid selected from the group consisting of tyrosine, phenylalanine and tryptophan.

10. A mouse heterozygous for a targeted amyloid precursor protein-encoding gene comprising: (1) a human Aβ peptide-encoding sequence in place of the endogenous murine Aβ peptide-encoding sequence; and (2) at least one Swedish FAD mutation.

11. The mouse of claim 10, wherein said human Aβ peptide-encoding sequence encodes an arginine residue at Aβ position 5 (APP770 position 676), a tyrosine residue at Aβ position 10 (APP770 position 681), and an histidine residue at Aβ position 13 (APP770 position 684).

12. The mouse of claim 10, wherein said amyloid precursor protein-encoding gene encodes a leucine residue at APP770 position 671.

13. The mouse of claim 10, wherein said amyloid precursor protein-encoding gene encodes an asparagine residue at APP770 position 670, and a leucine residue at APP770 position 671.

14. The mouse of claim 10, wherein said human Aβ peptide is produced by endogenous mechanisms for processing amyloid precursor proteins.

15. The mouse of claim 14, wherein the amount of native murine Aβ peptide present in any given tissue is between 30% and 80% of the amount present in the corresponding tissue of a wild-type control mouse.

16. The mouse of claim 14, wherein the amount of native murine Aβ peptide present in any given tissue is less than 10% of the amount present in the corresponding tissue of a wild-type control mouse.

17. The mouse of claim 14, wherein the rate of amyloid precursor protein synthesis in any given tissue is substantially the same as the rate of amyloid precursor protein synthesis in the corresponding tissue of a wild-type control mouse.

18. The mouse of claim 14, wherein production of said human Aβ peptide in any given tissue is greater than the production of native murine Aβ peptide in the corresponding tissue of a wild-type control mouse.

19. A mouse heterozygous for a targeted amyloid precursor protein-encoding gene comprising: (1) a human Aβ peptide-encoding sequence in place of the endogenous murine Aβ peptide-encoding sequence; and (2) a sequence encoding, at the APP codon corresponding to murine APP770 position 671, an amino acid selected from the group consisting of tyrosine, phenylalanine and tryptophan.

20. A method for screening chemical compounds for the ability to inhibit *in vivo* processing of amyloid precursor protein to yield the human Aβ peptide, said method comprising the steps of:

(a) administering said chemical compounds to a mouse homozygous for a targeted amyloid precursor protein-encoding gene comprising: (1) a human Aβ peptide-encoding sequence in place of the native murine Aβ peptide-encoding sequence; and (2) at least one Swedish FAD mutation; and

(b) measuring the relative amounts of amyloidogenic and nonamyloidogenic processing of amyloid precursor protein in a sample from said mouse, at an appropriate interval after administration of said chemical compounds, wherein said sample is selected from the group consisting of brain tissue, non-brain tissue, and body fluids.

21. The mouse of claim 1, wherein said mouse lacks at least one normal SOD-1 allele.

22. The mouse of claim 2, wherein said mouse lacks both normal SOD-1 alleles.

23. The mouse of claim 6, wherein said mouse has a phenotype that includes a reduced amount of Cu/Zn SOD protein expression.

24. The mouse of claim 23, wherein said mouse produces substantially no measurable amount of Cu/Zn SOD protein.

25. The method of claim 20, wherein said mouse of lacks at least one normal SOD-1 allele.

26. The method of claim 20, wherein said mouse produces substantially no measurable amount of Cu/Zn SOD protein.

Figure 1

Prototype Gene Targeting Strategy

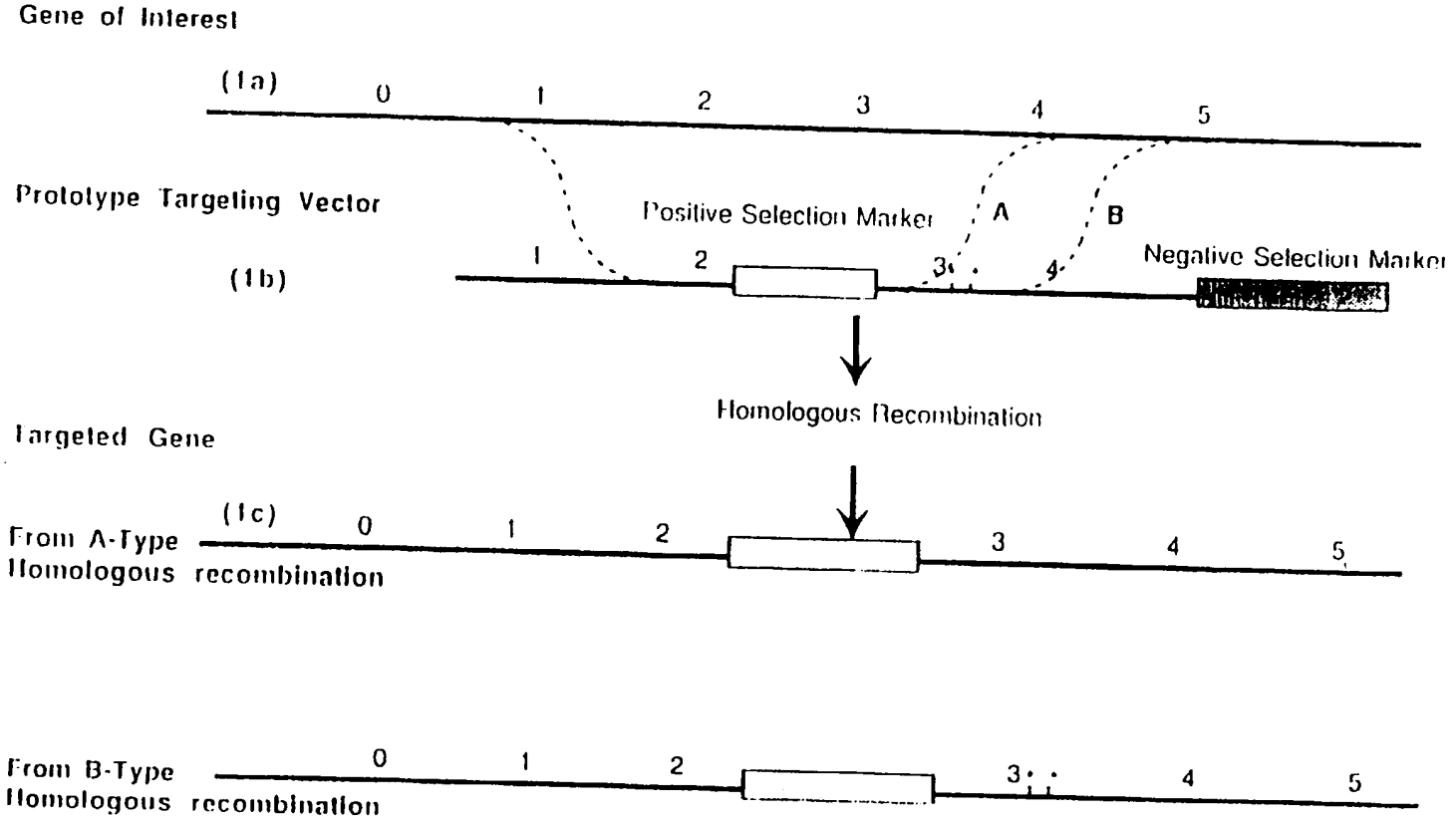
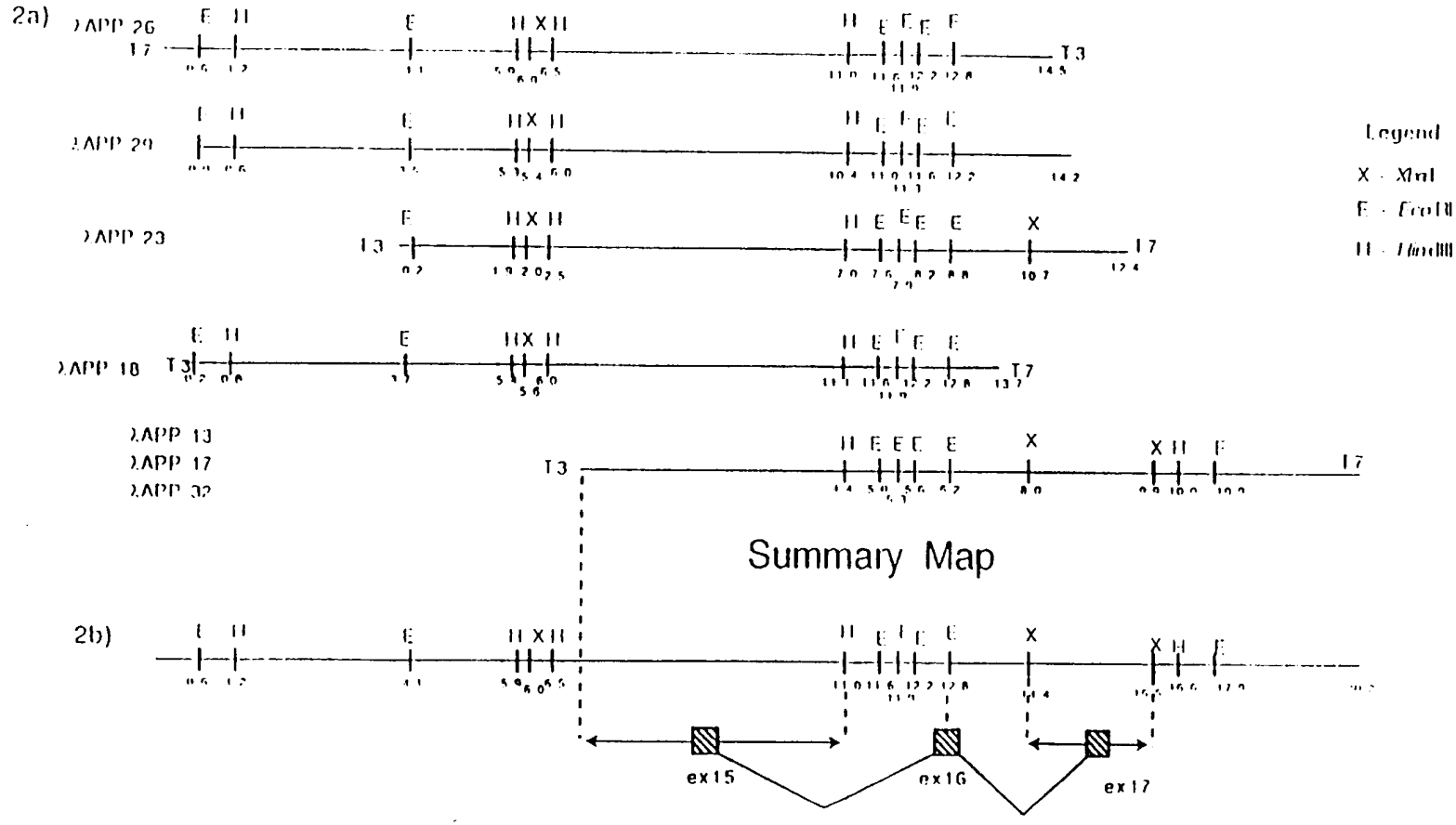


Figure 2

Mouse APP Exon 16 Region Genomic Clone Maps



Restriction Mapping With FLASH™ Gene Mapping Kit

Figure 3

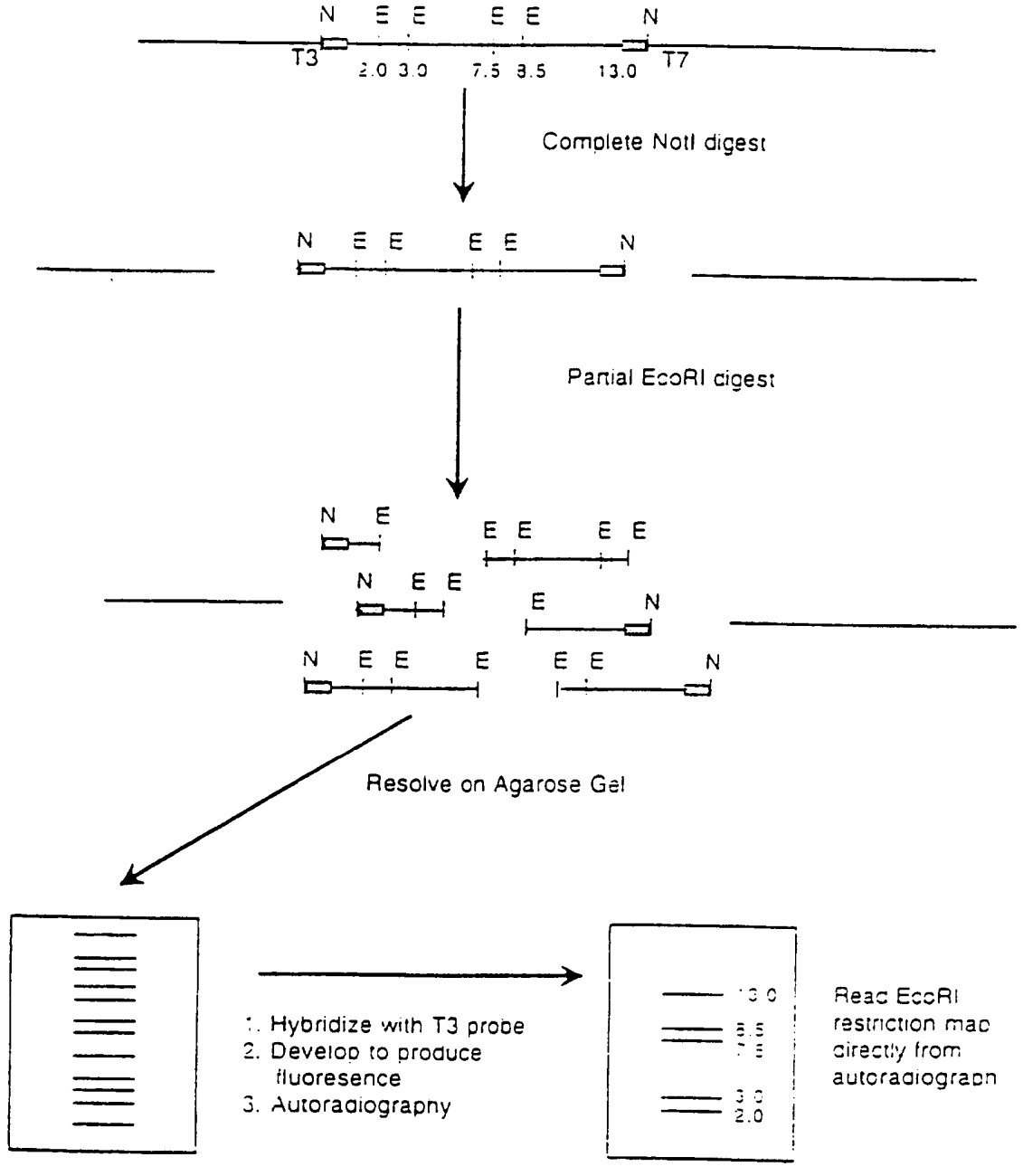


Figure 4

Strategy Used to Place Exons 15, 16, and 17 on APP Restriction Map

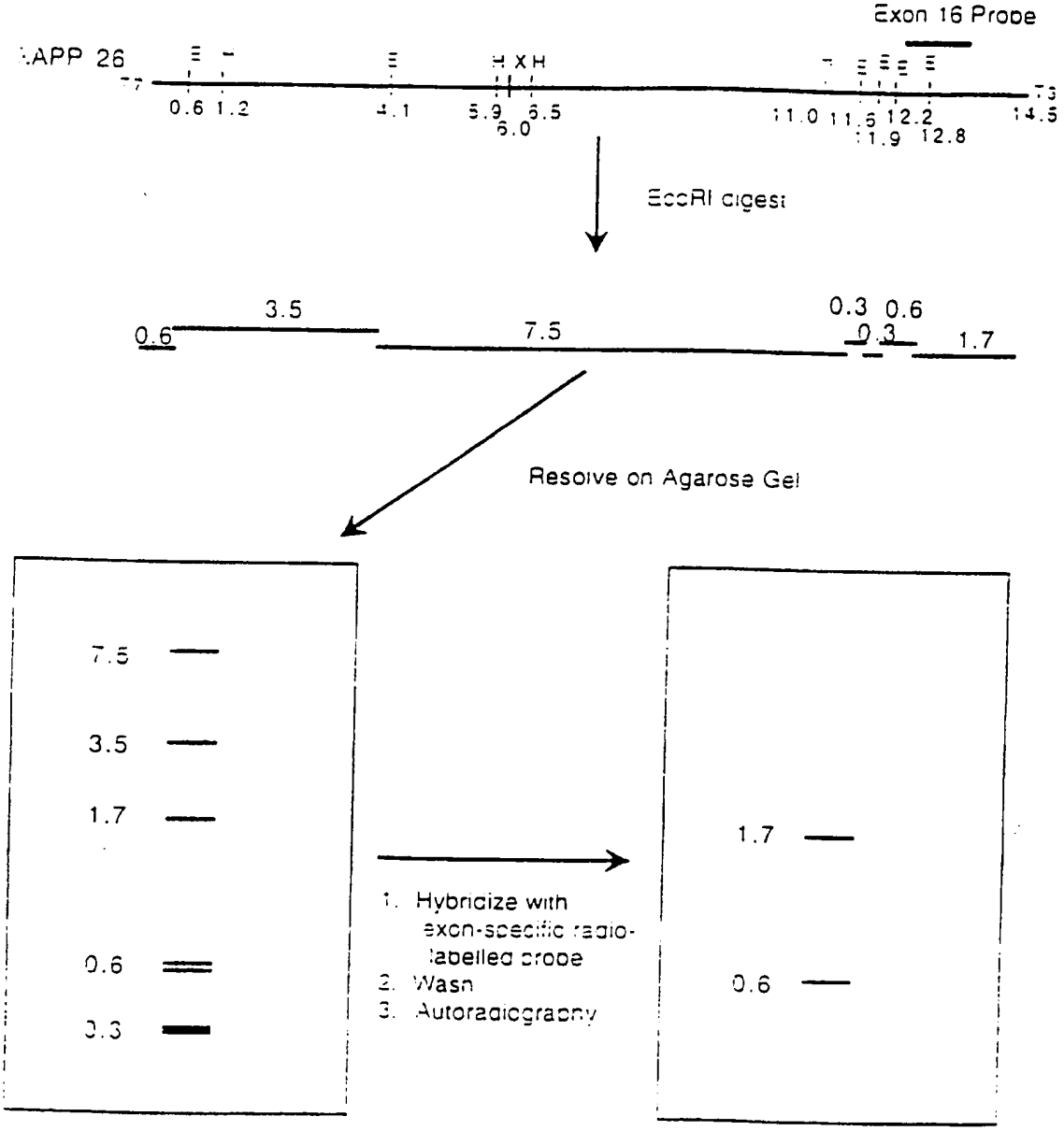
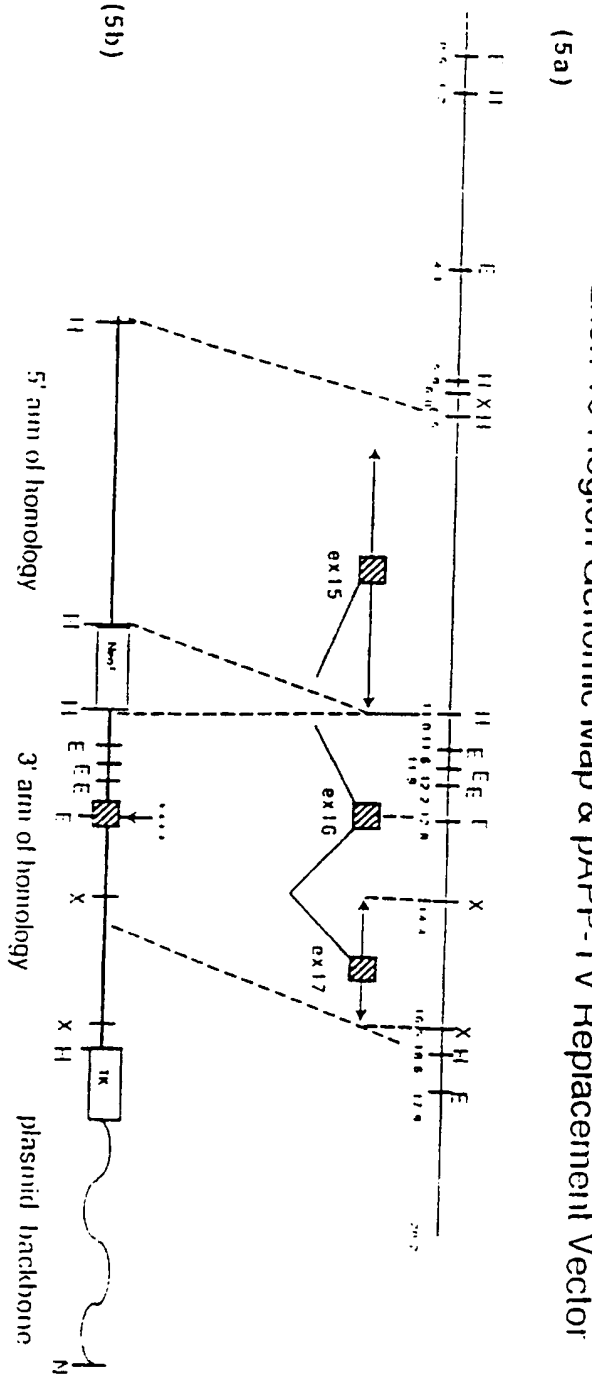


Figure 5


(5a)
APP Exon 16 Region Genomic Map & pAPP-TV Replacement Vector
ex15
ex16
ex17
(5b)
5' arm of homology
3' arm of homology
plasmid backbone
Legend
EcoRI - E
HindIII - H
XbaI - X
Not I - N

pPNTlox^2 Synthesis

Figure 6

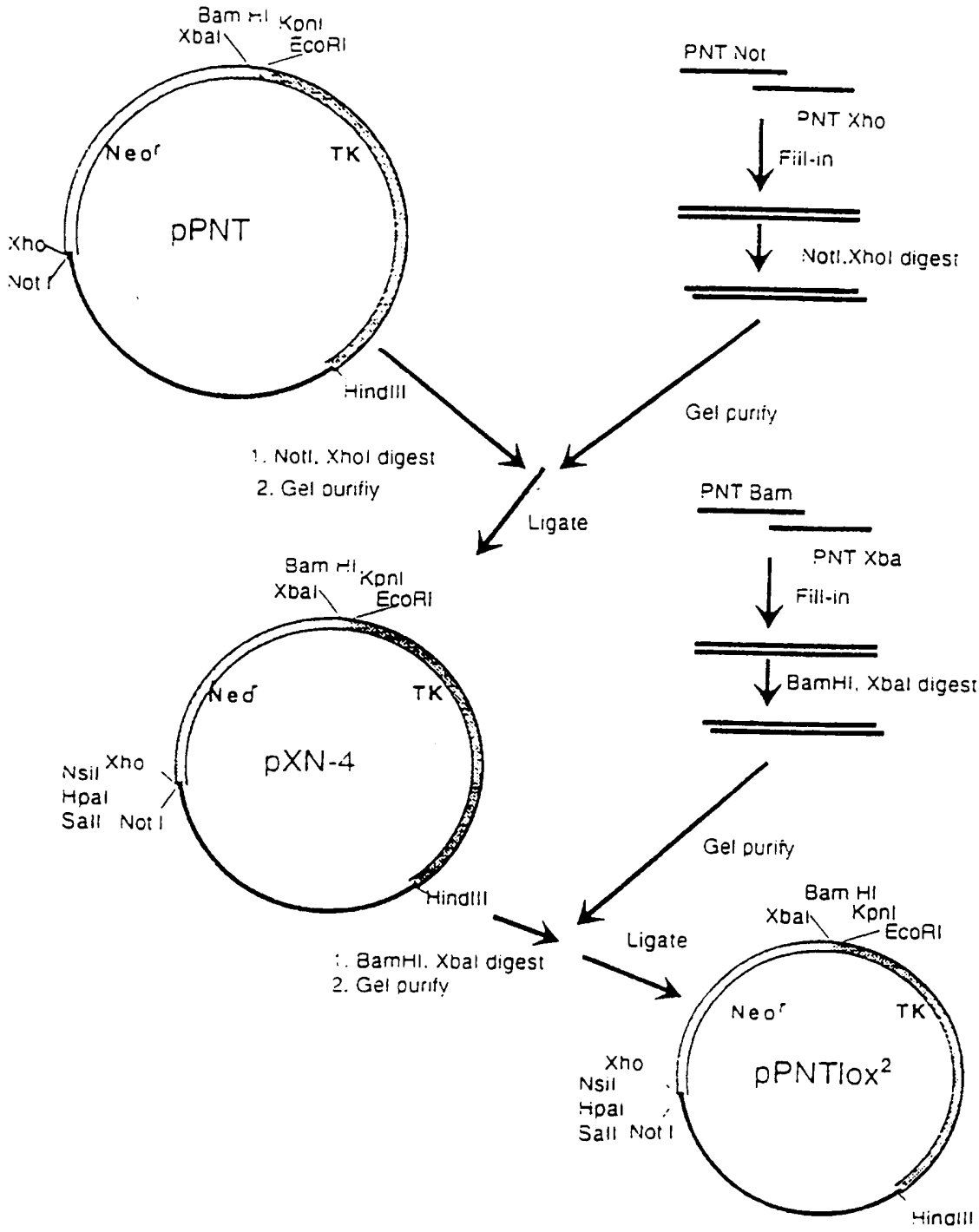
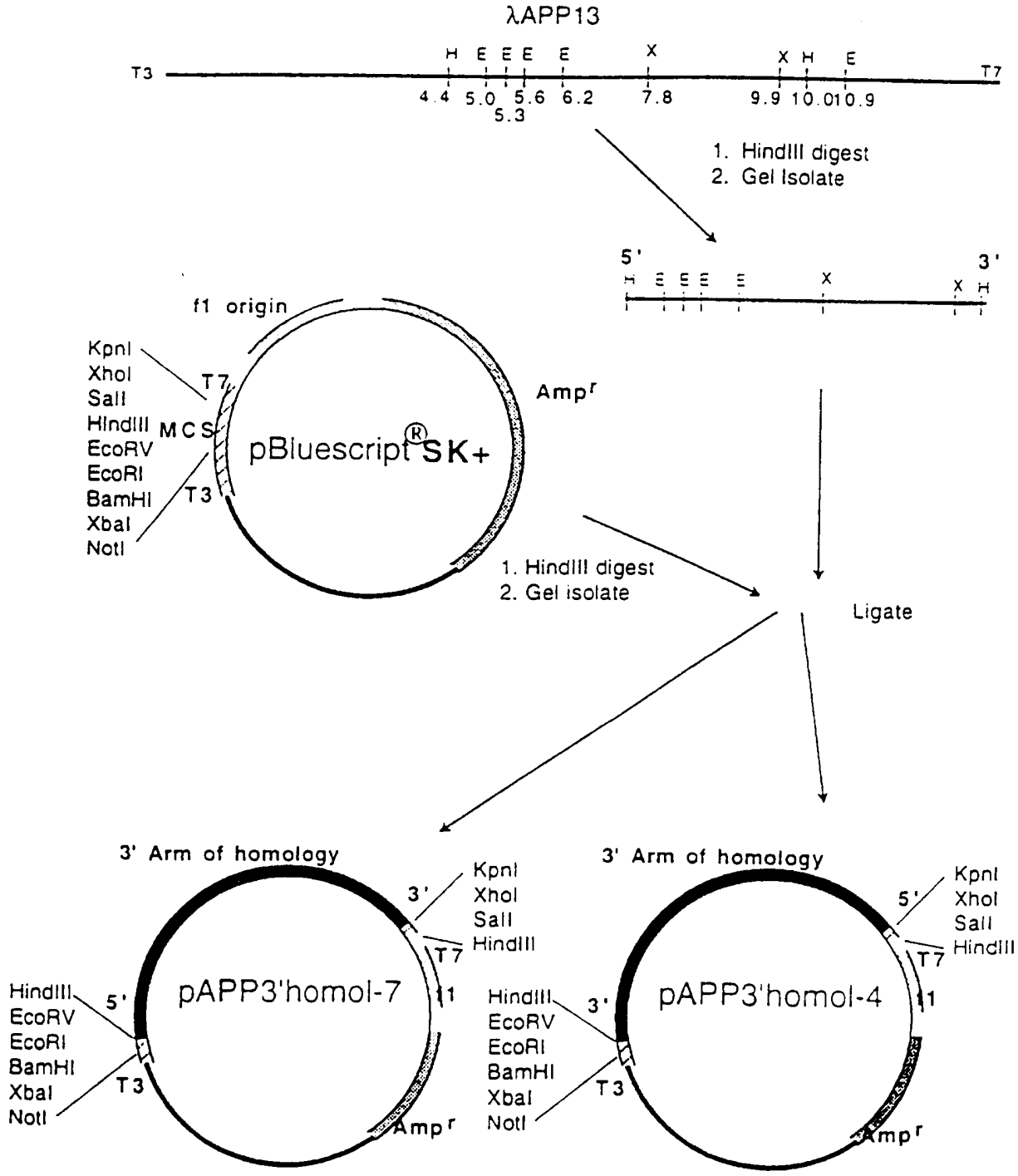


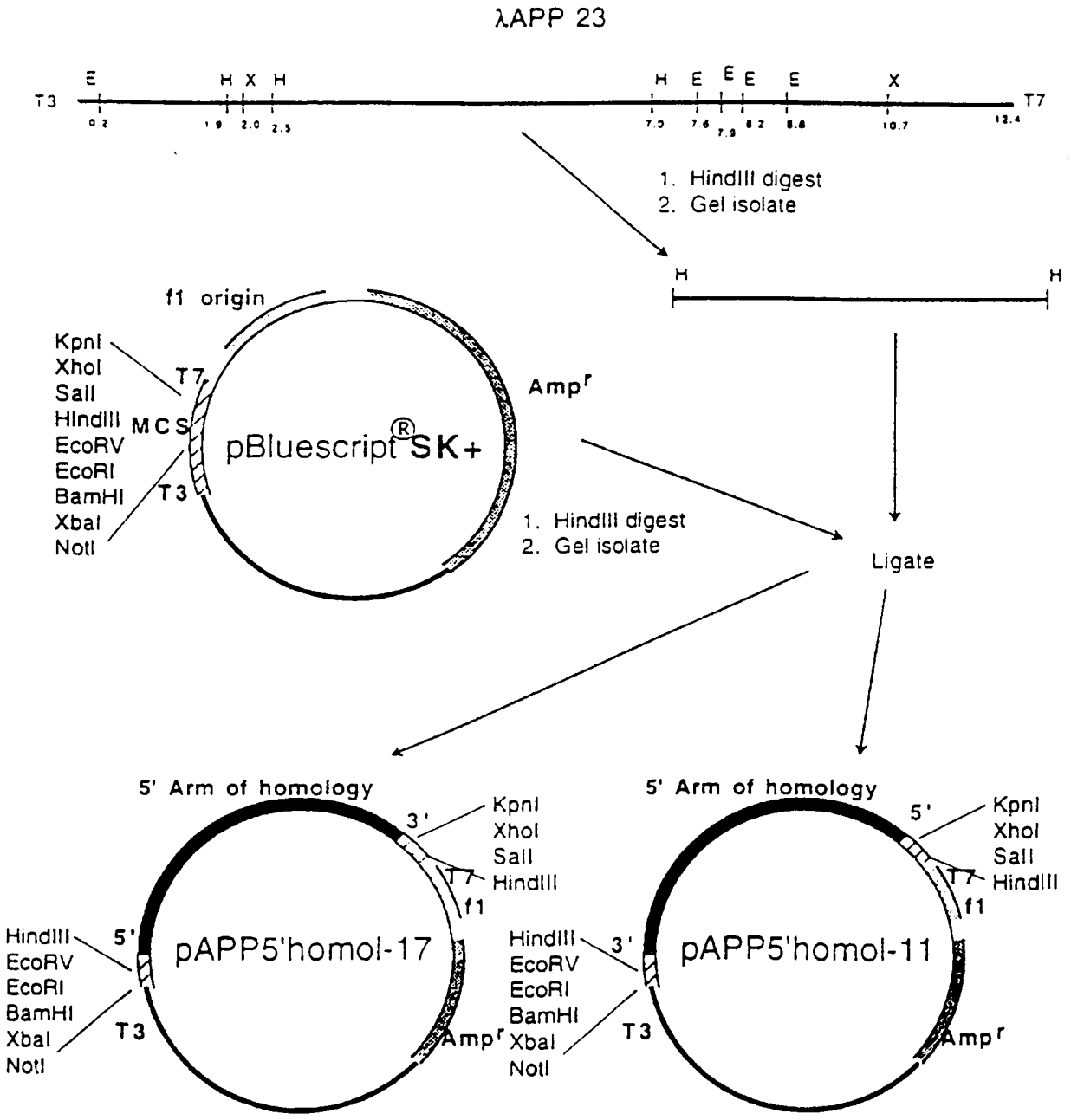
Figure 7

pAPP3'homol-4, -7 Construction



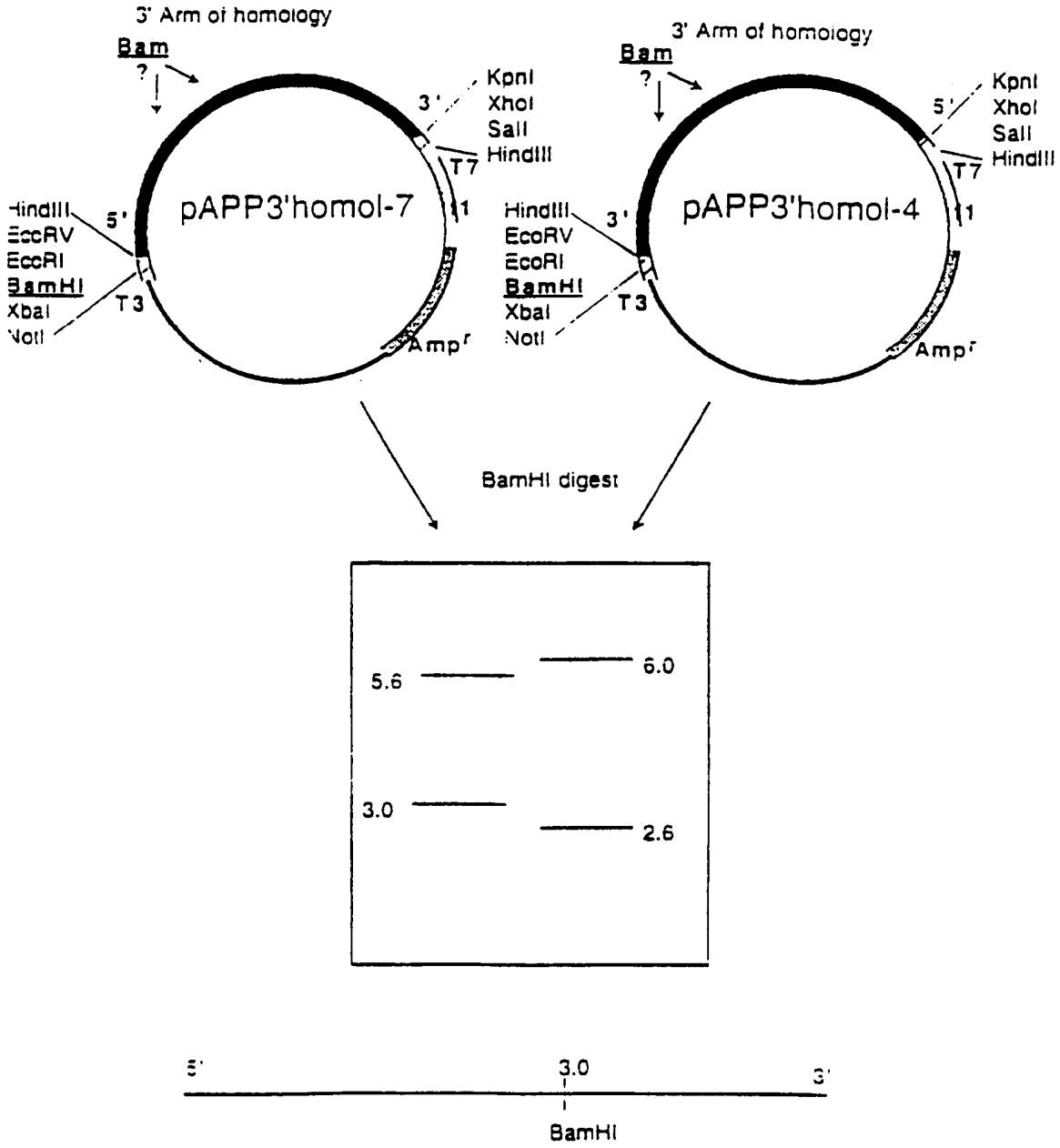
pAPP5'homol-11, -17 Construction

Figure 8



Example of Restriction Mapping the 3' Arm of Homology

Figure 9



Detailed Restriction Map Summary For APP 3' and 5' Arms of Homology

Figure 10

5' Arm

5' Homol: present but not mapped:
ClaI HincII

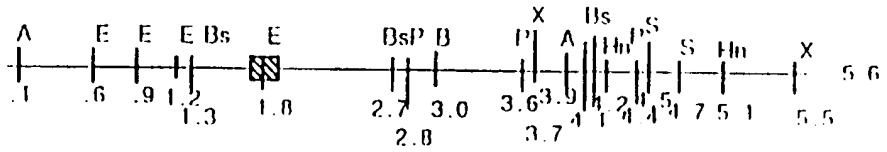
5' Homol: *No sites for:*

Bam HI	Apa I
EcoRV	Sal I
Xho I	

3' Arm

3' Homol: present but not mapped:
NsiI

3' Homol: No sites for:
Apa, Cla EcoRV Kpn, Sal ,
Xho, Eag, Spe, Sac I Hpal

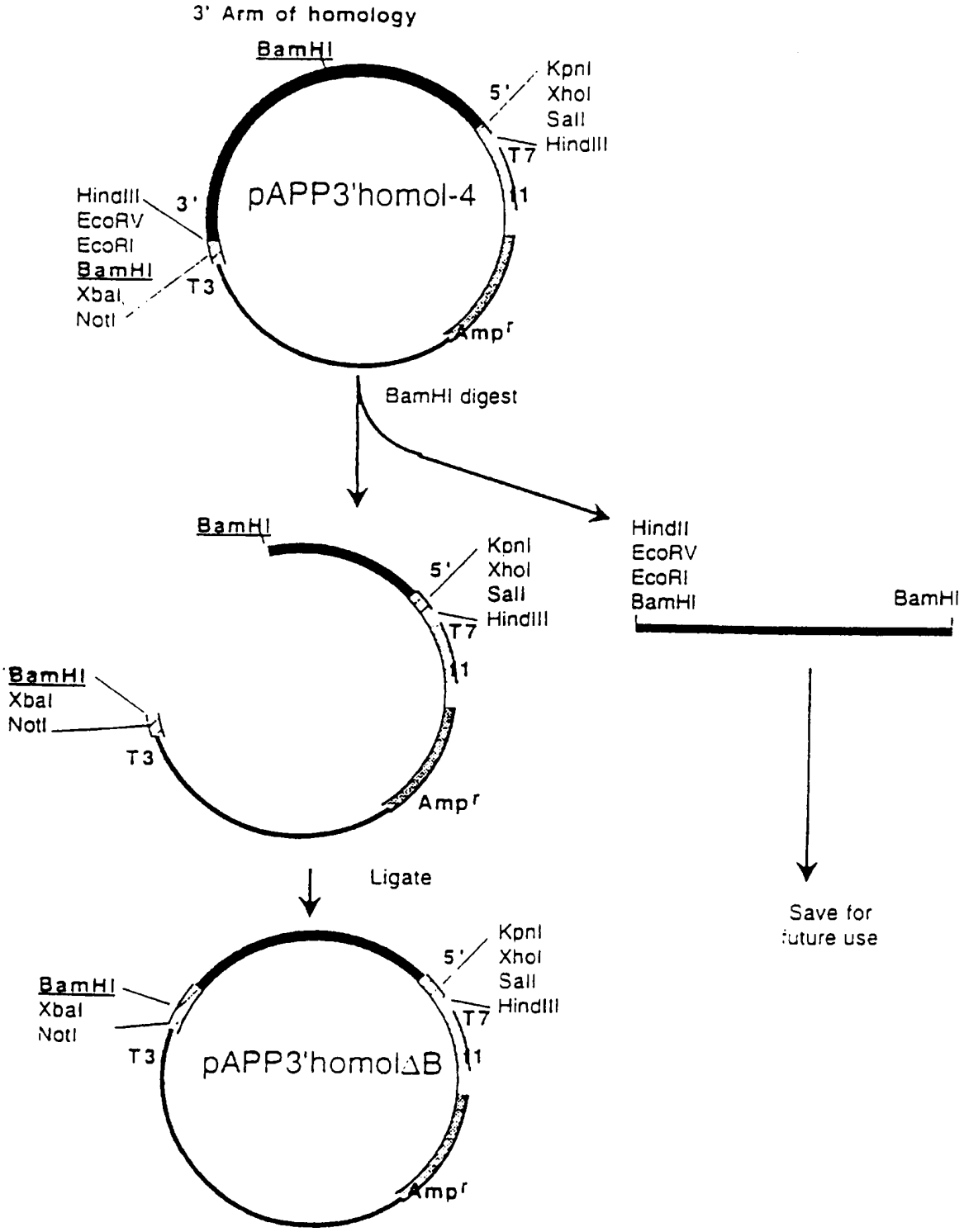


Legend

E - EcoRI
H - HindIII
X - XbaI
Hn - HincII
P - PstI
A - AccI
B - BamHI
Bs - BstXI
S - SmaI

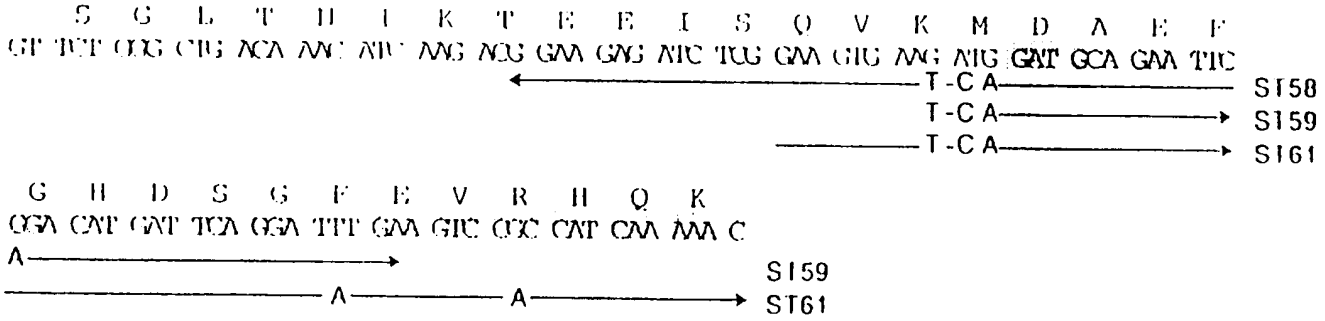
pAPP3'homolΔB Construction

Figure 11



Mouse APP Exon 16 Mutagenesis

Figure 12



pAPP3'homolΔB-NL Construction

Figure 13

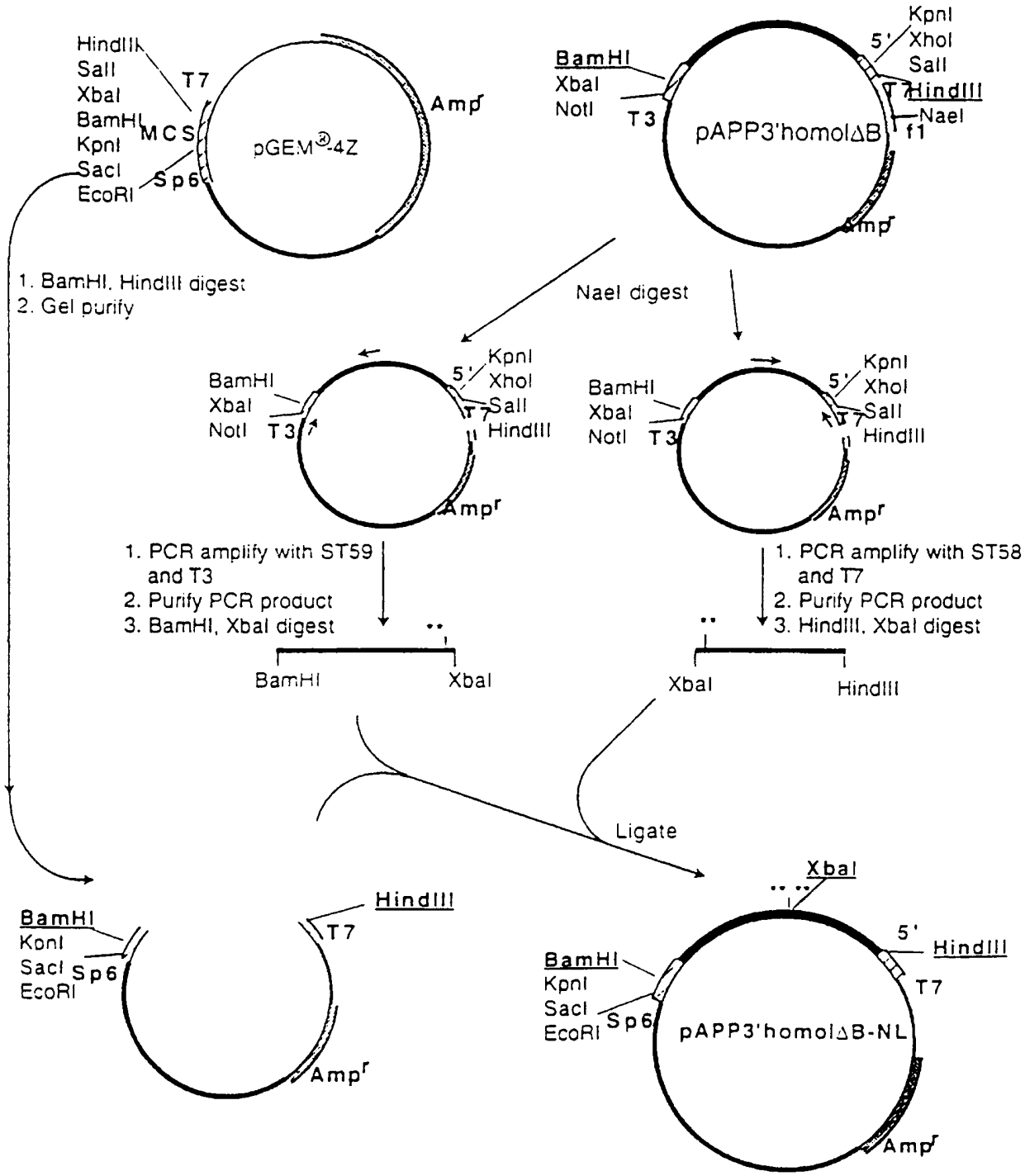
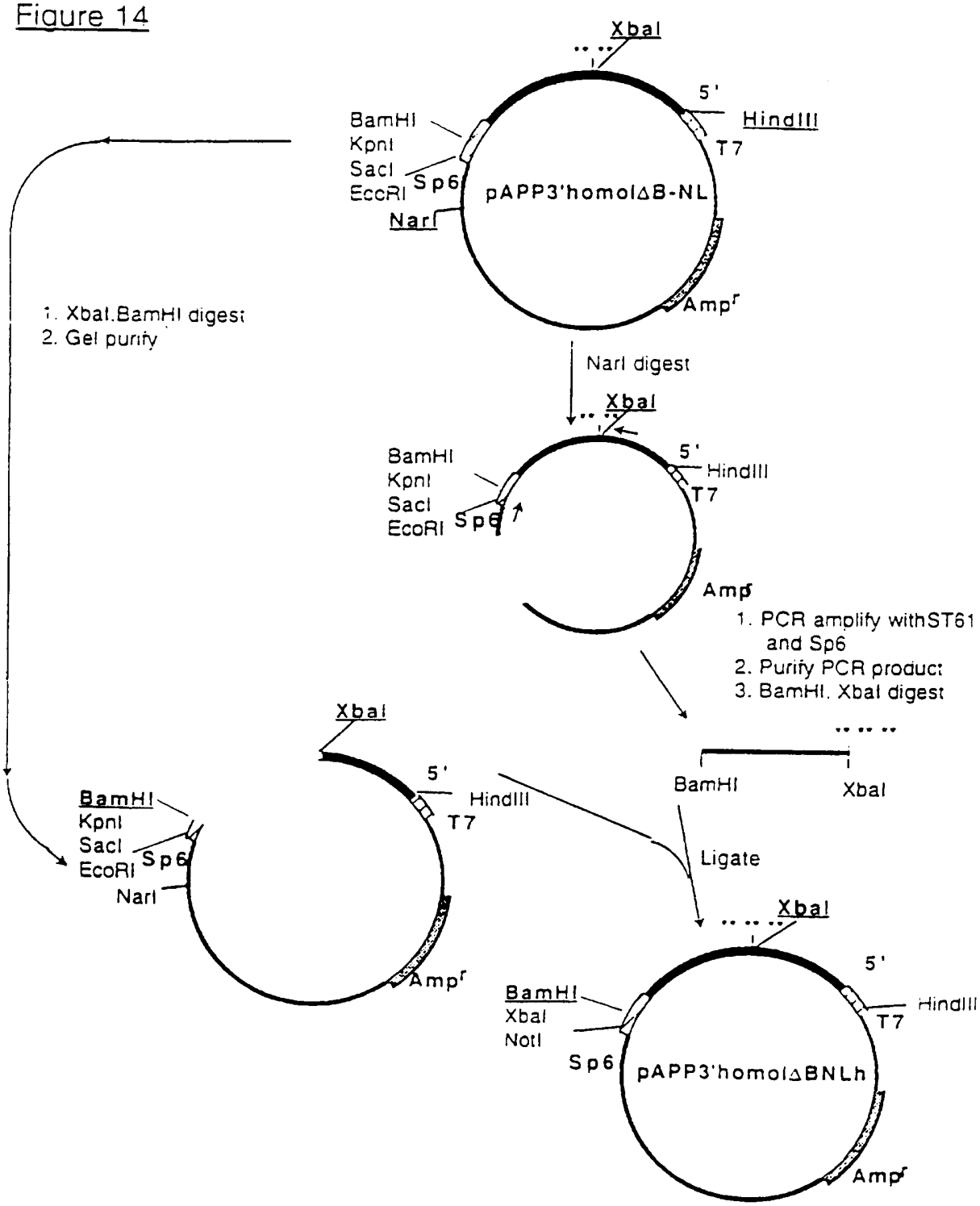


Figure 14


pAPP3'homolΔB-NLh Construction
XbaI
5'
HindIII
T7
BamHI
KpnI
SacI
EcoRI
Sp6
NarI
pAPP3'homolΔB-NL
Ampr
1. XbaI.BamHI digest
2. Gel purify
NarI digest
1. PCR amplify withST61 and Sp6
2. Purify PCR product
3. BamHI. XbaI digest
Ligate
pAPP3'homolΔBNLh
NotI

p4Z3'homolNL-h Construction

Figure 15

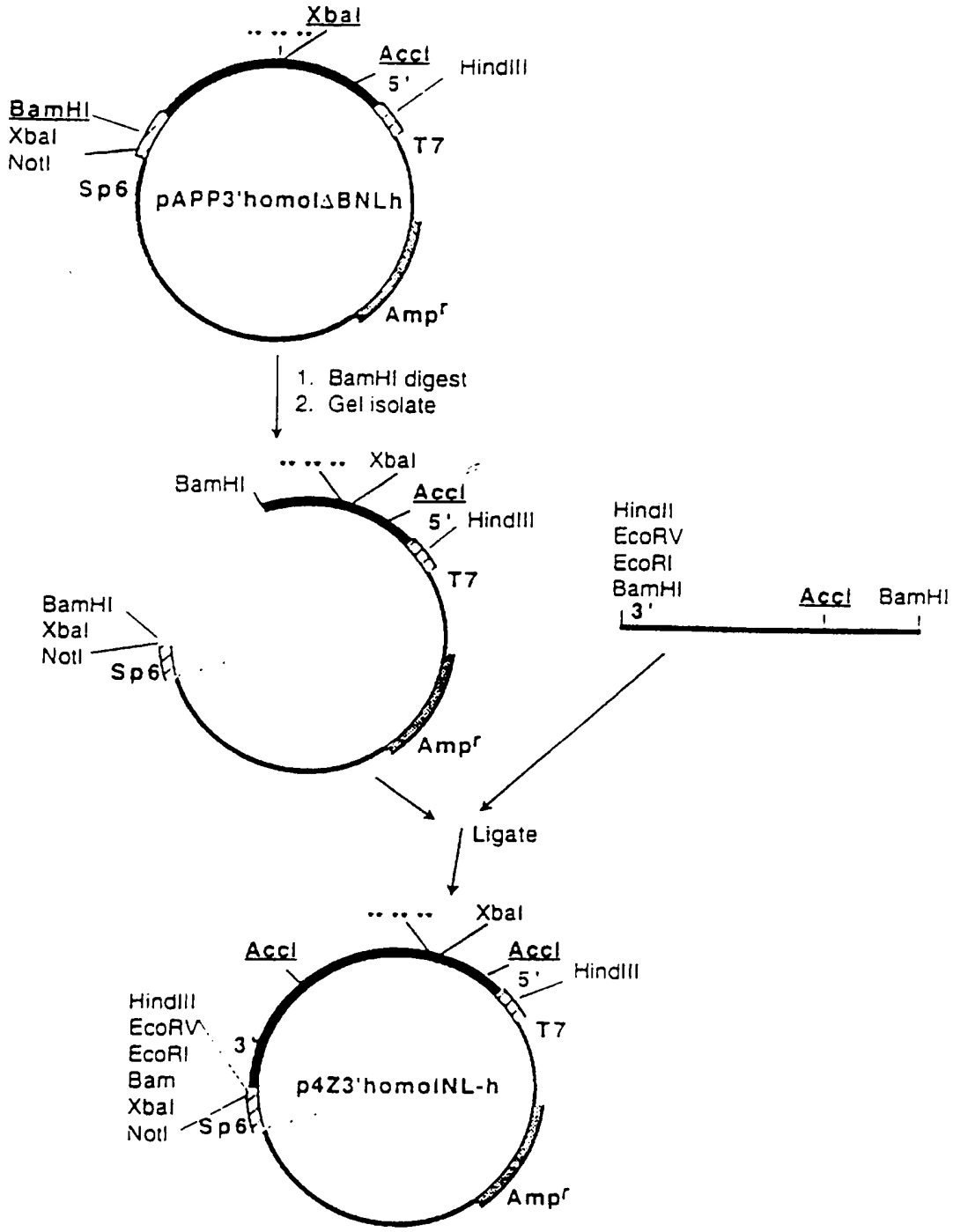
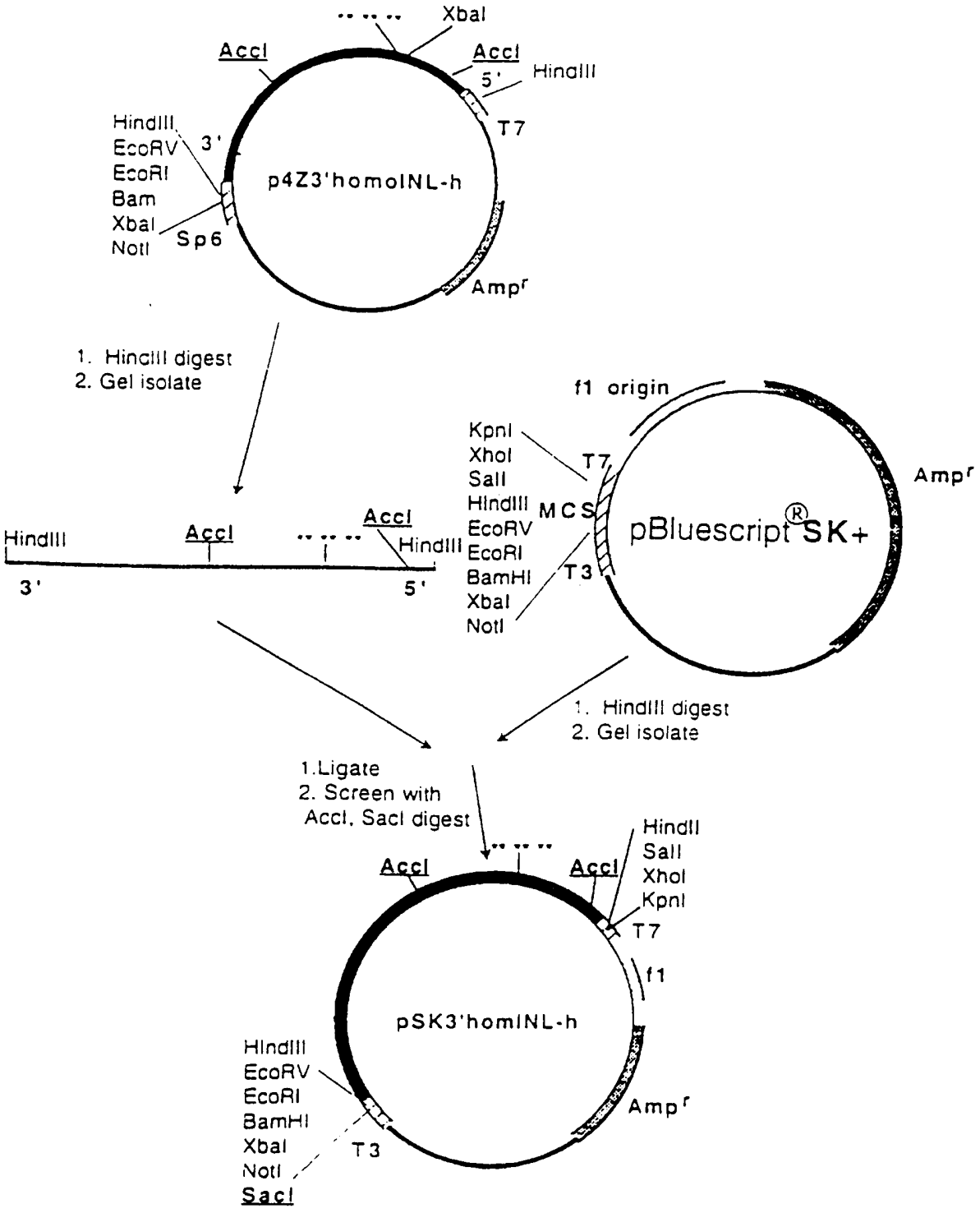
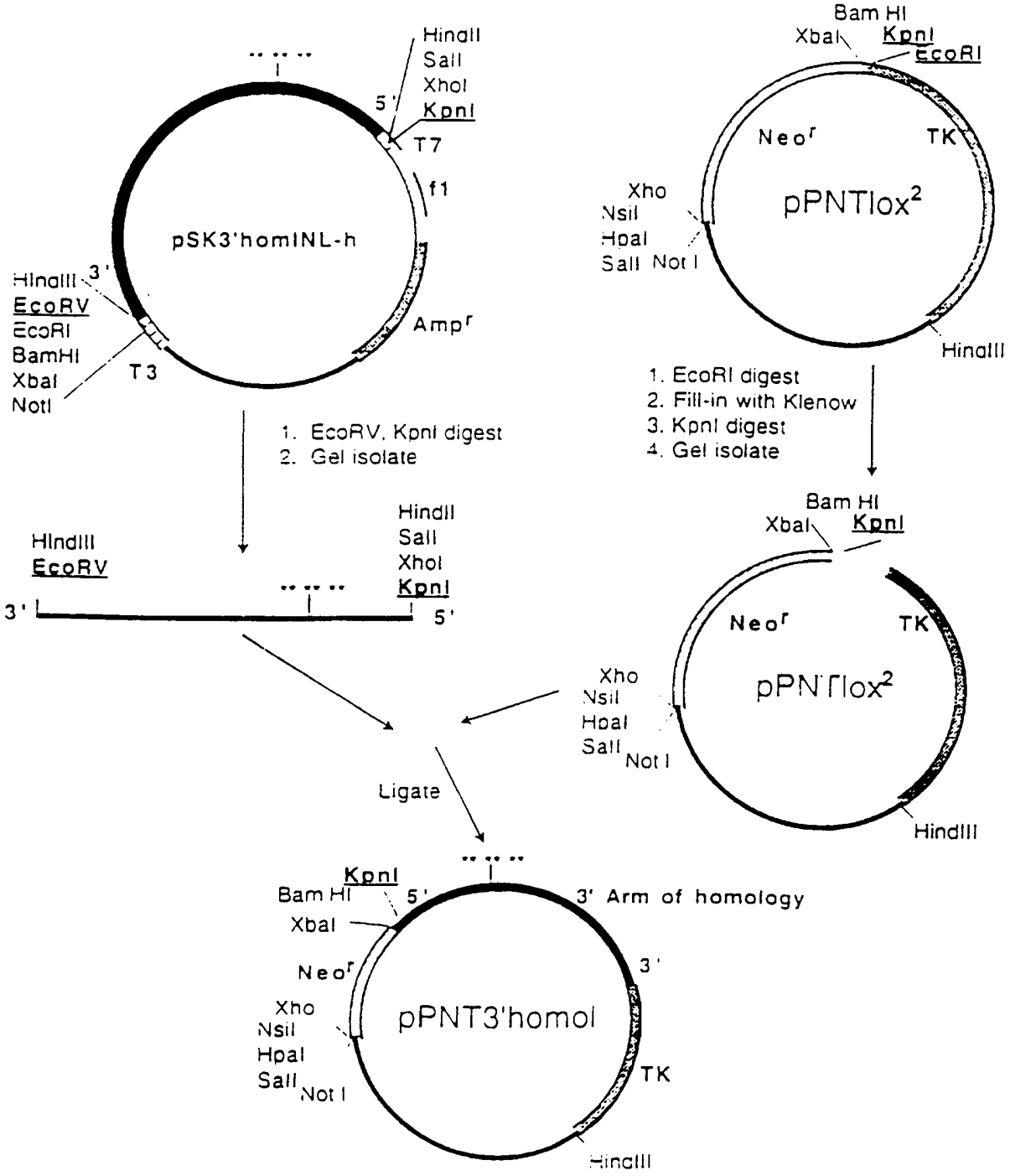


Figure 16


pSK3'homolNL-h Construction
Xbal
AccI
AccI
5'
HindIII
T7
HindIII
EcoRV
EcoRI
Bam
Xbal
NotI
3'
Sp6
p4Z3'homolNL-h
Ampr
1. HincIII digest
2. Gel isolate
f1 origin
KpnI
XhoI
SalI
HindIII
EcoRV
EcoRI
BamHI
Xbal
NotI
T7
MCS
T3
pBluescript® SK+
Ampr
HindIII
AccI
AccI
HindIII
3'
5'
1. HindIII digest
2. Gel isolate
1.Ligate
2. Screen with
AccI, SacI digest
AccI
AccI
HindII
SalI
XhoI
KpnI
T7
f1
pSK3'homlNL-h
HIndIII
EcoRV
EcoRI
BamHI
Xbal
NotI
SacI
T3
Ampr

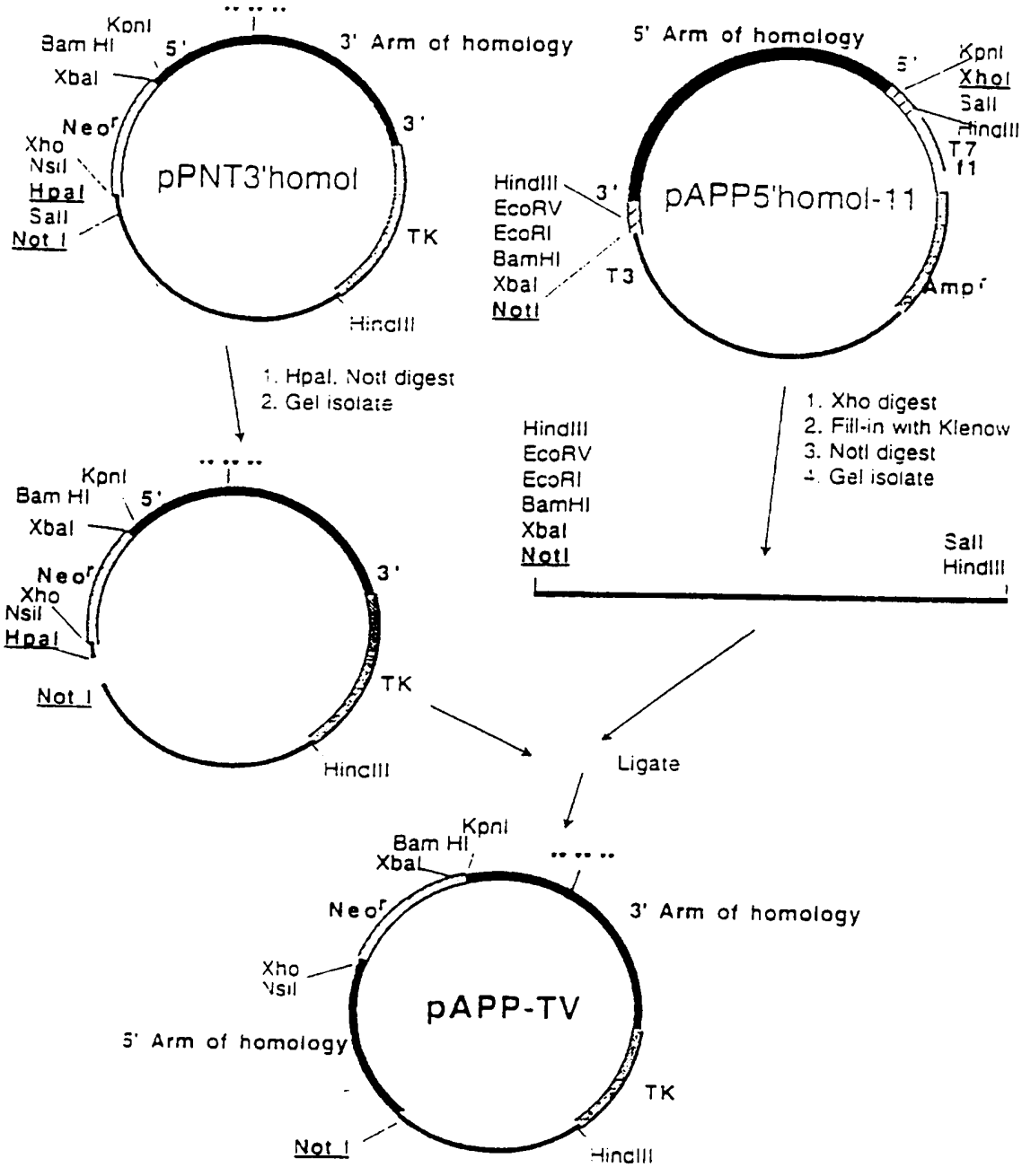
pPNT3'homol Construction

Figure 17



pAPP-TV Construction

Figure 18



Strategy to detect homologous recombination within mouse APP

Figure 19

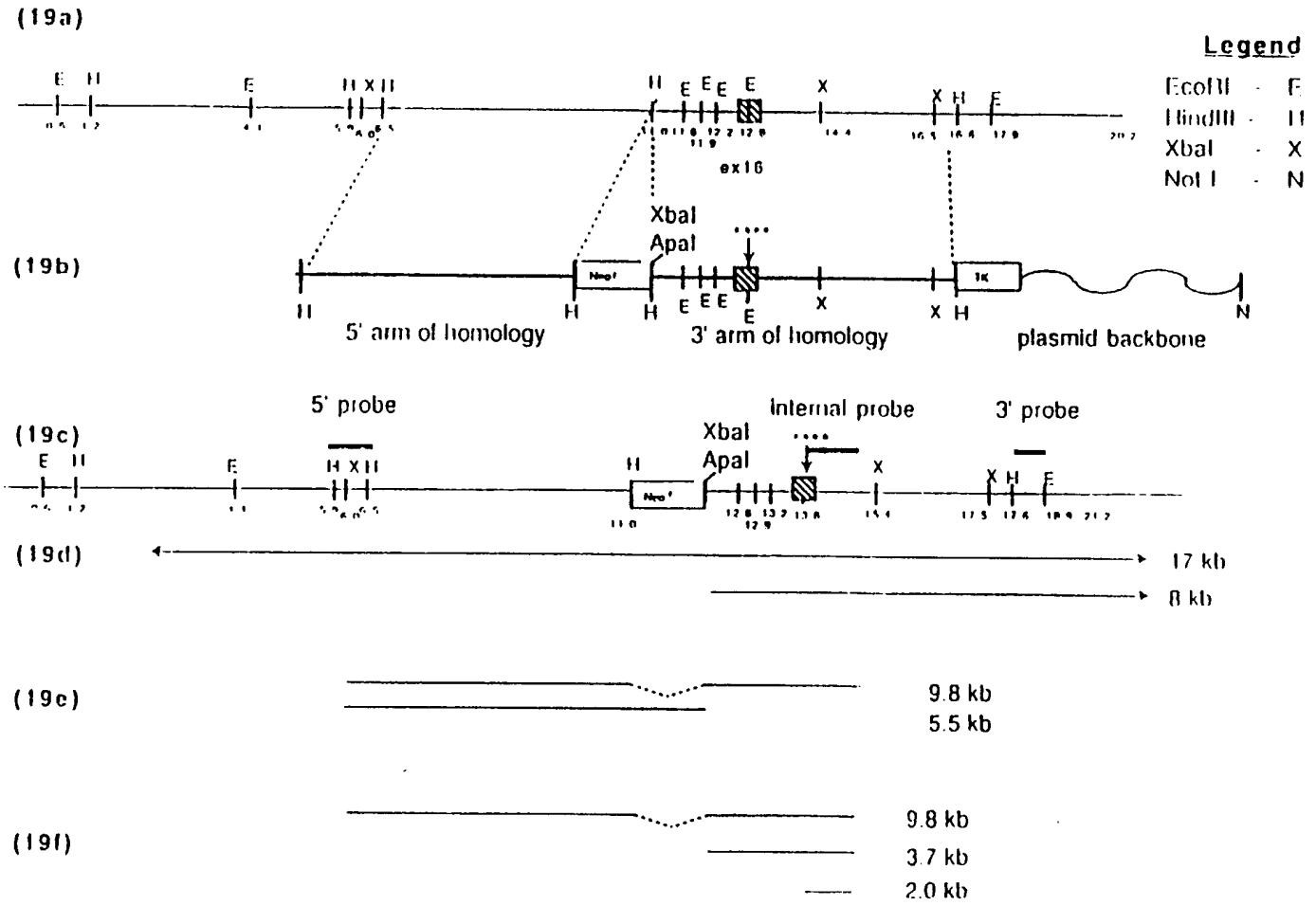


FIGURE 20

Schematic of APP, Relevant Carboxyl-terminal derivatives (CTDs) and Antibodies

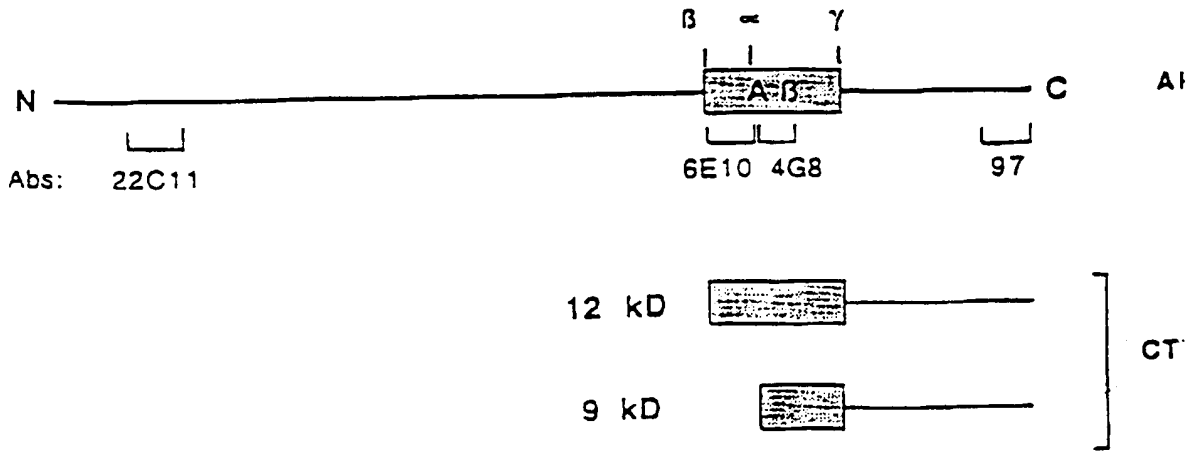


FIGURE 21

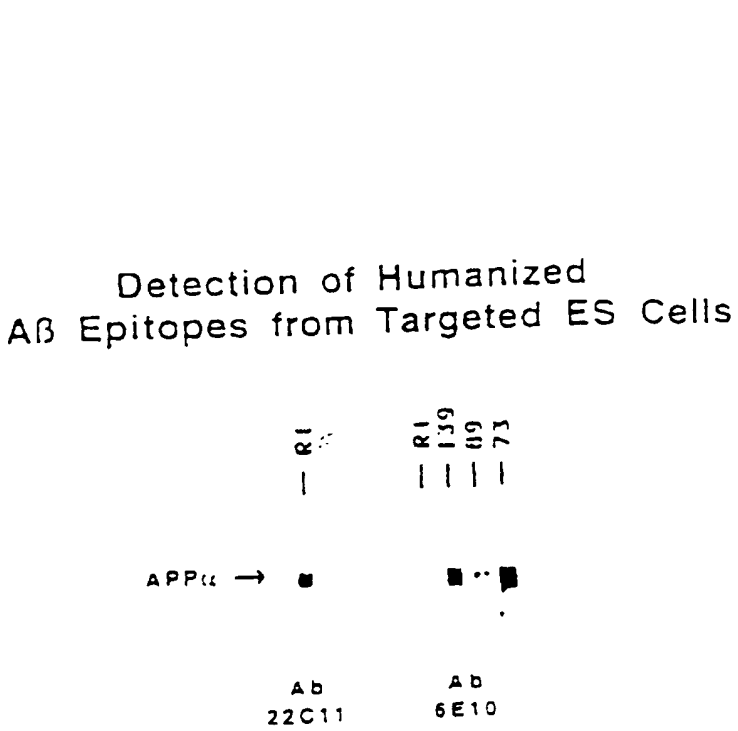
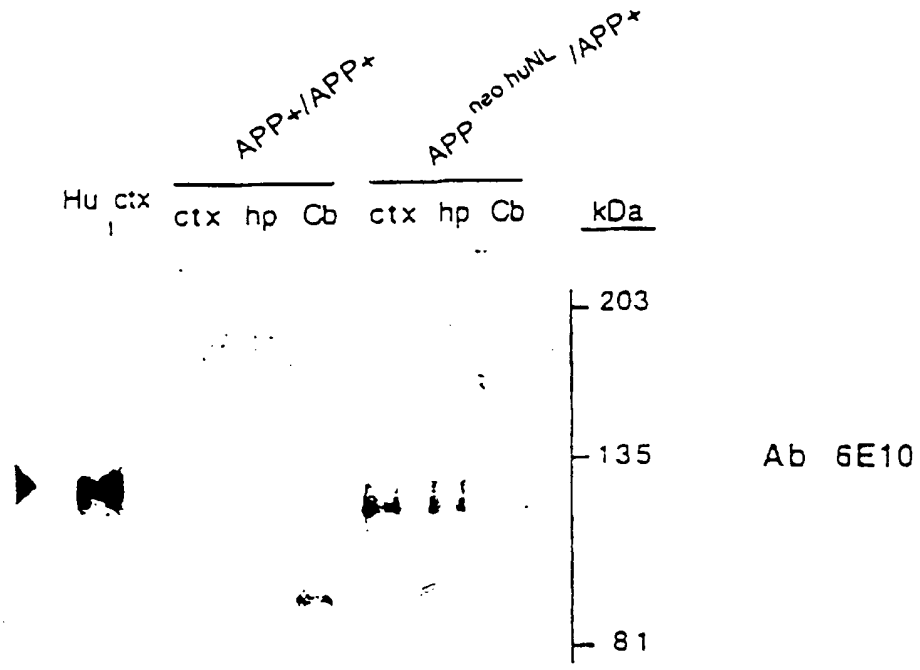

Detection of Humanized
Aß Epitopes from Targeted ES Cells
RI
RI
139
89
73
APPα →
Ab
22C11
Ab
6E10

FIGURE 22

Humanized APP is Expressed in Targeted Mouse Brain



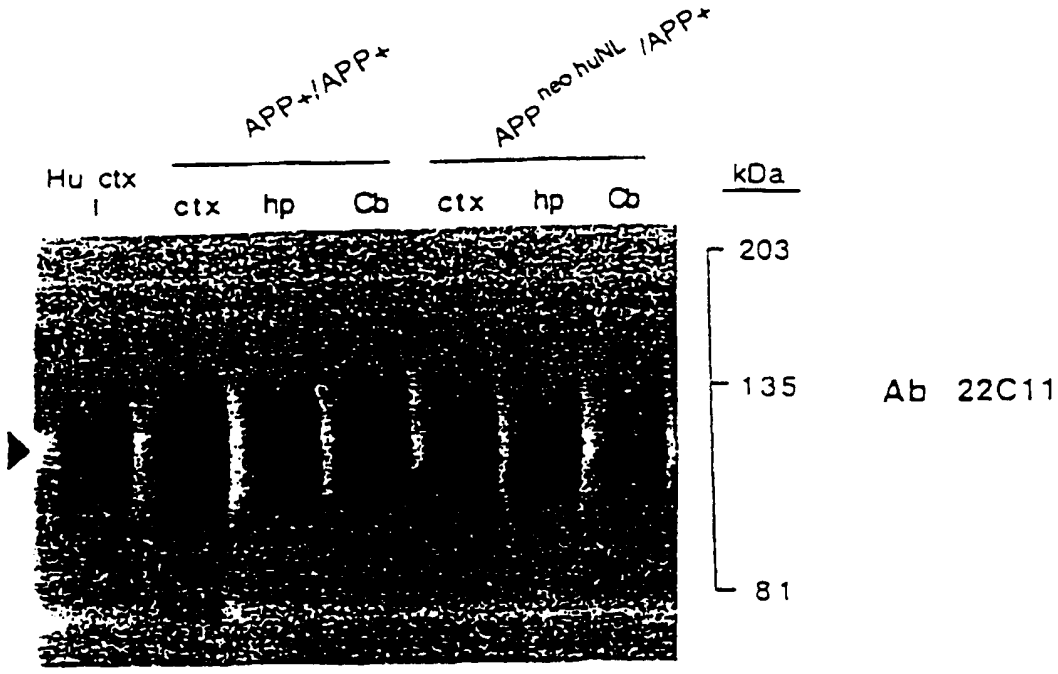
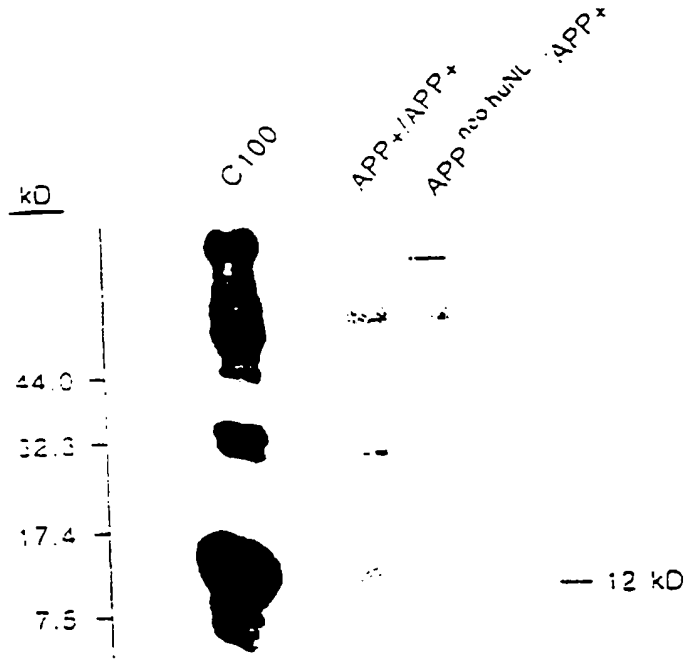
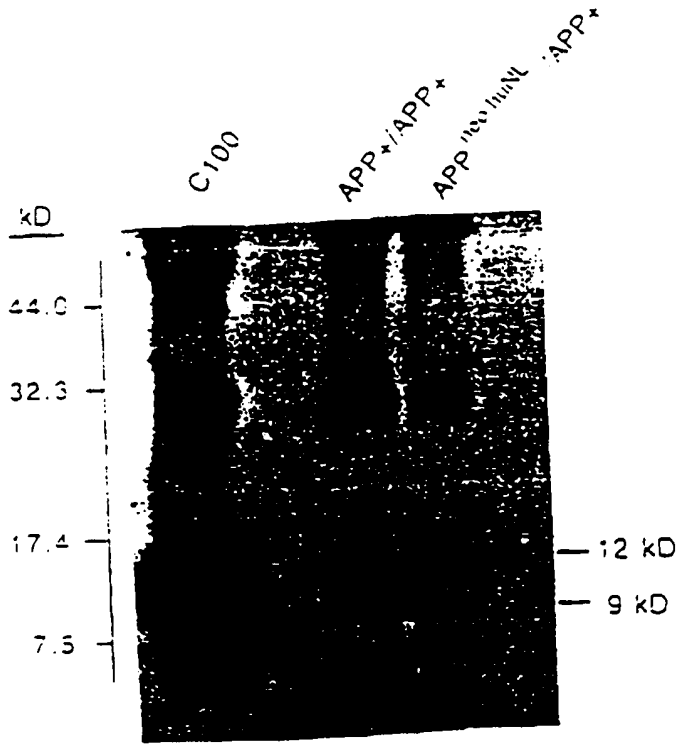


FIGURE 23

Detection of 12 kD and 9 kD Carboxyl-Terminal Derivatives of APP in Targeted Mouse Brain



Ab 6E10



Ab 4G8

Fig. 24 A

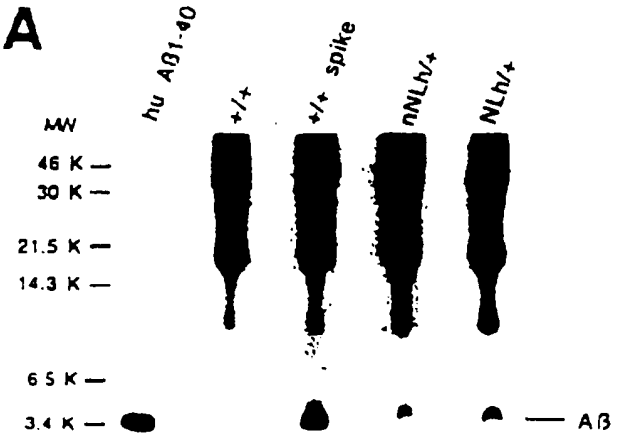


Fig. 24B

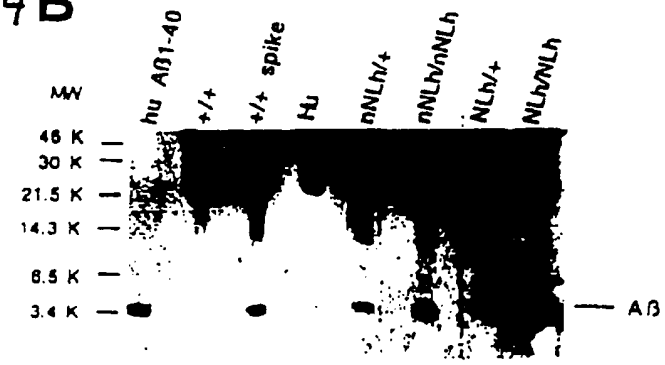
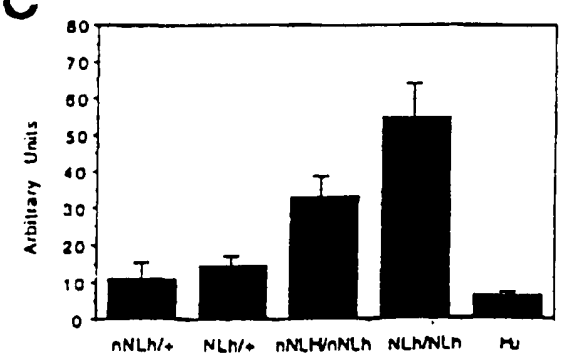


Fig. 24C



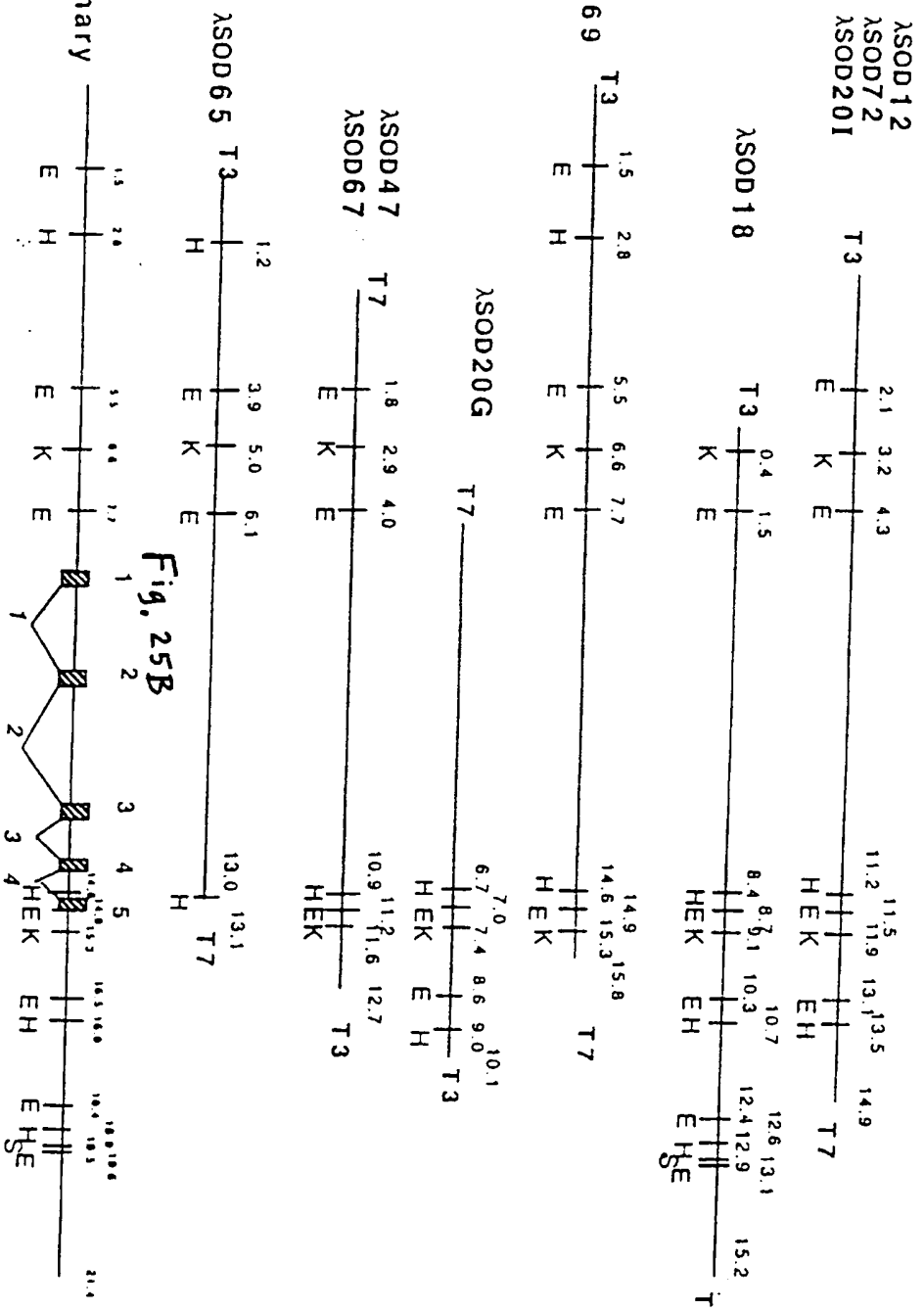


Fig. 25A

Fig. 25B

Fig. 26

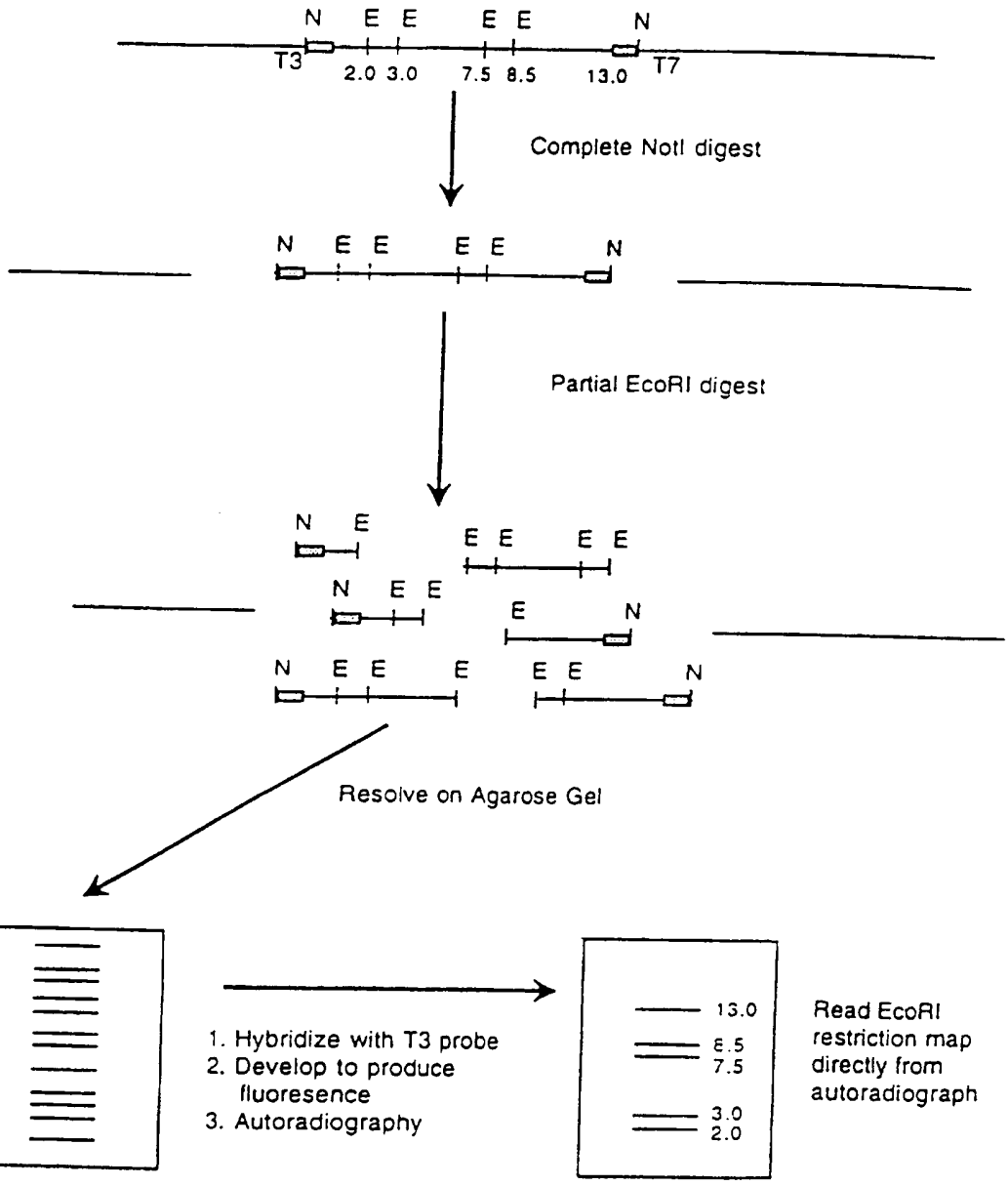

N E E E E N
T3
2.0 3.0 7.5 8.5 13.0
T7
Complete NotI digest
Partial EcoRI digest
Resolve on Agarose Gel
1. Hybridize with T3 probe
2. Develop to produce fluoresence
3. Autoradiography
13.0
8.5
7.5
3.0
2.0
Read EcoRI restriction map directly from autoradiograph

Fig. 27

SOD Genomic Map & pSOD-TV Deletion Vector

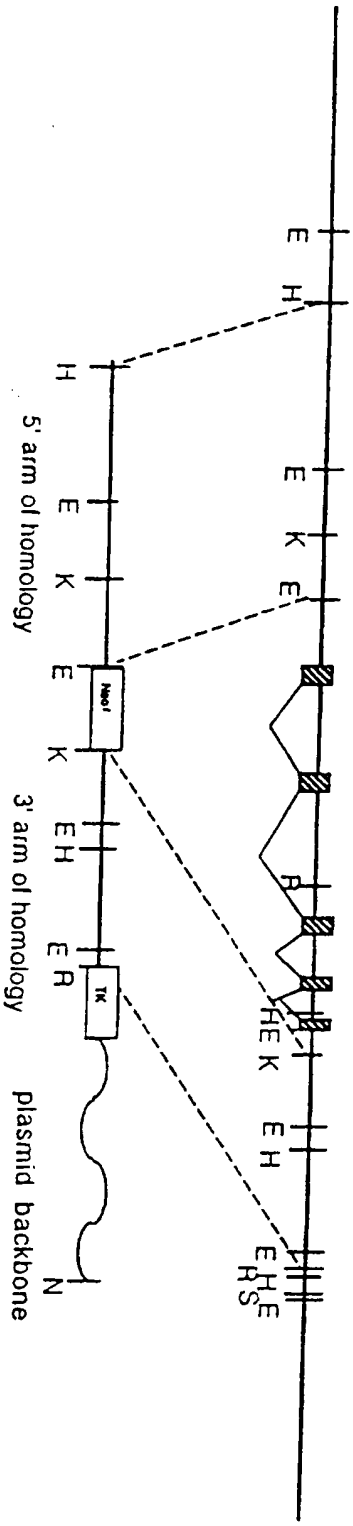


Fig. 28

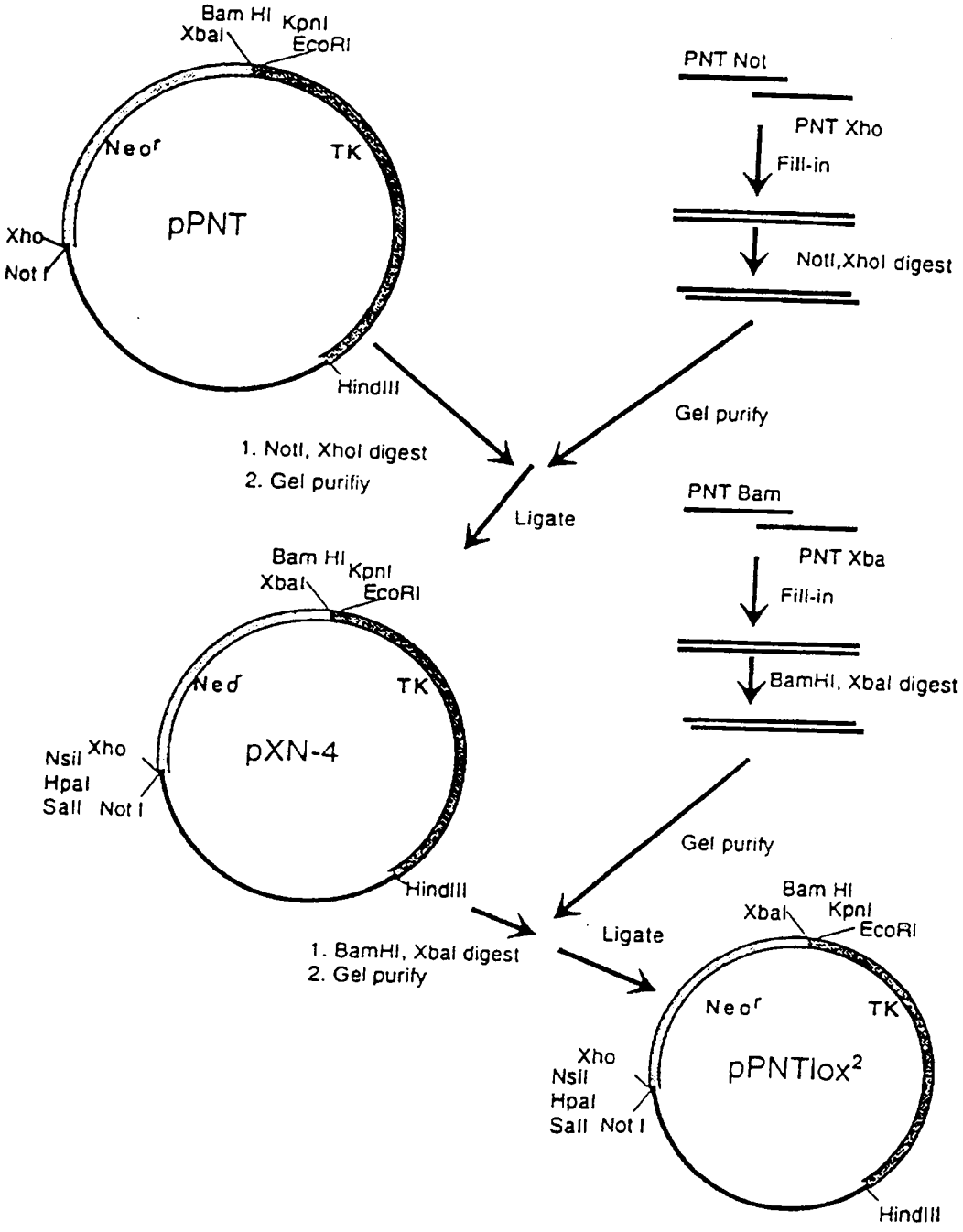

Bam HI
KpnI
XbaI
EcoRI
Neo^r
TK
pPNT
Xho
Not I
HindIII
1. NotI, XhoI digest
2. Gel purifiy
PNT Not
PNT Xho
Fill-in
NotI,XhoI digest
Gel purify
Ligate
PNT Bam
PNT Xba
Fill-in
BamHI, XbaI digest
Gel purify
Bam HI
XbaI
KpnI
EcoRI
Neo^r
TK
pXN-4
NsiI
Xho
HpaI
SalI
Not I
HindIII
1. BamHI, XbaI digest
2. Gel purify
Ligate
Bam HI
XbaI
KpnI
EcoRI
Neo^r
TK
pPNTlox2
Xho
NsiI
HpaI
SalI
Not I
HindIII

Fig. 29

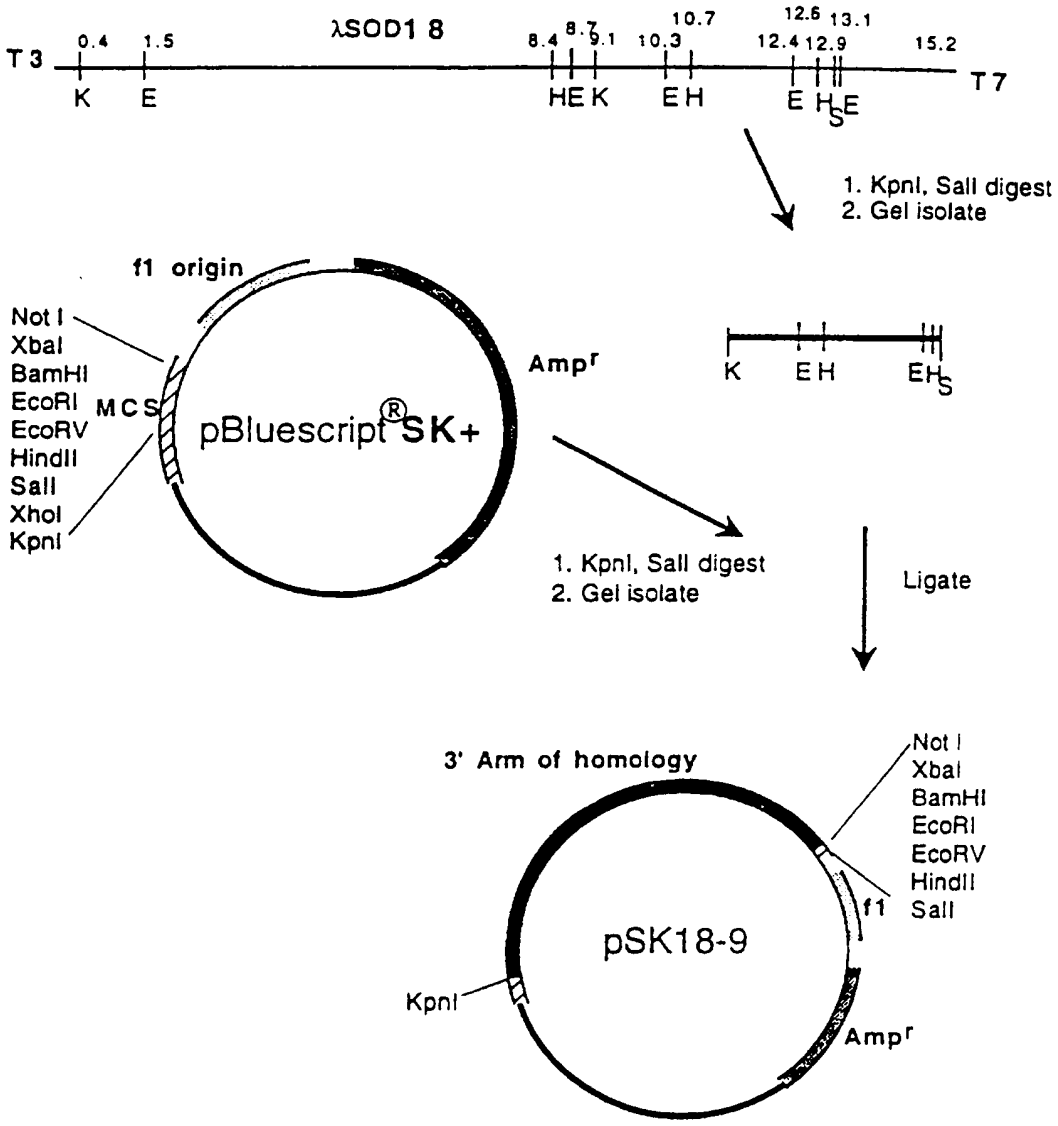

λSOD1 8
T3
T7
0.4
1.5
8.4
8.7
9.1
10.3
10.7
12.4
12.6
12.9
13.1
15.2
K
E
HE K
E H
E H S E
1. KpnI, SalI digest
2. Gel isolate
f1 origin
Not I
XbaI
BamHI
EcoRI
EcoRV
HindII
SalI
XhoI
KpnI
MCS
pBluescript® SK+
Ampr
K
E H
E H S
1. KpnI, SalI digest
2. Gel isolate
Ligate
3' Arm of homology
Not I
XbaI
BamHI
EcoRI
EcoRV
HindII
SalI
f1
pSK18-9
KpnI
Ampr

Fig. 30

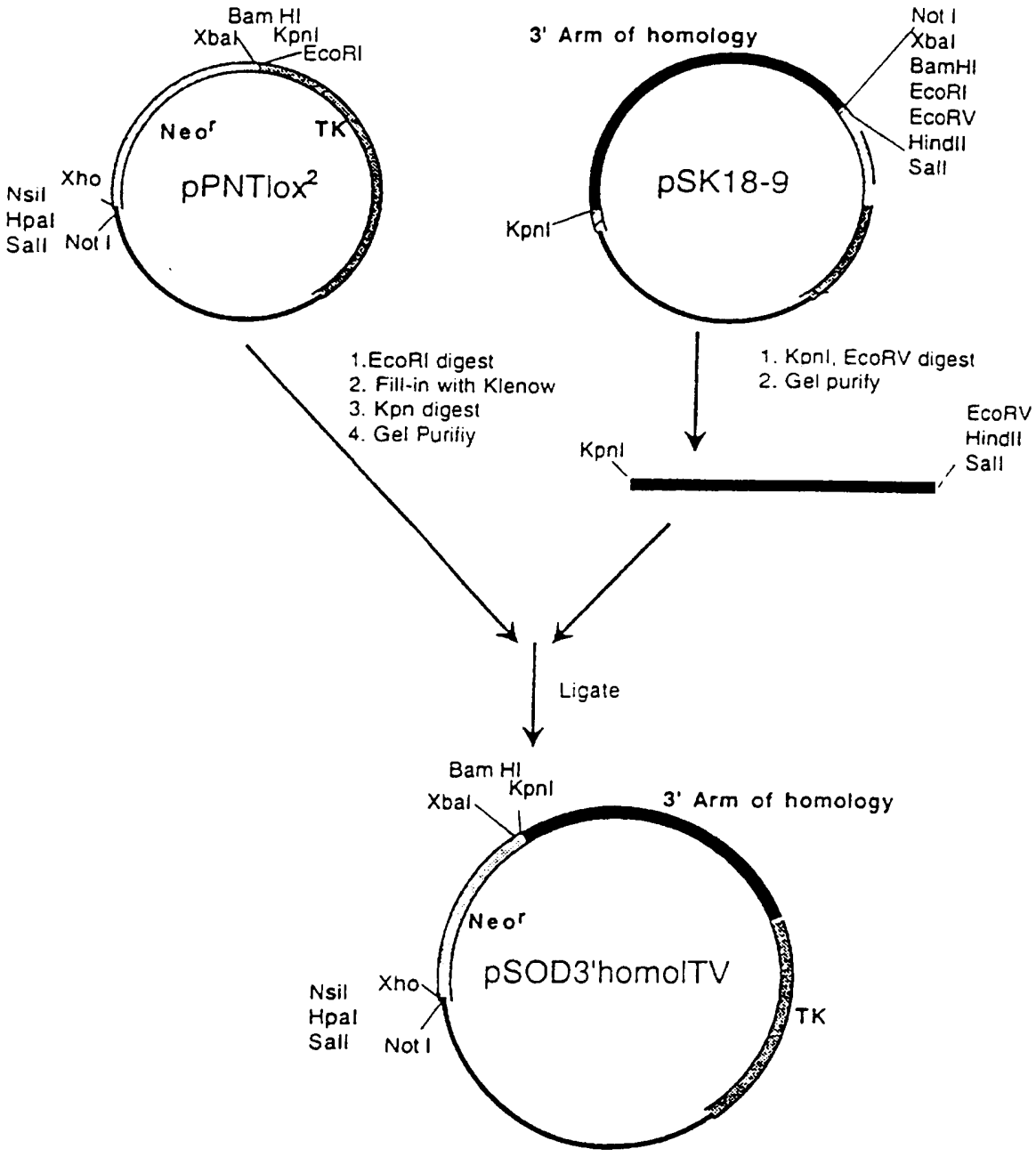

Bam HI
Xbal
KpnI
EcoRI
Neo^r
TK
pPNTlox^2
Xho
NsiI
HpaI
SalI
Not I
3' Arm of homology
Not I
Xbal
BamHI
EcoRI
EcoRV
HindII
SalI
pSK18-9
KpnI
1.EcoRI digest
2. Fill-in with Klenow
3. Kpn digest
4. Gel Purifiy
1. KpnI, EcoRV digest
2. Gel purify
KpnI
EcoRV
HindII
SalI
Ligate
Bam HI
Xbal
KpnI
3' Arm of homology
Neo^r
pSOD3'homolTV
Xho
NsiI
HpaI
SalI
Not I
TK

Fig. 31

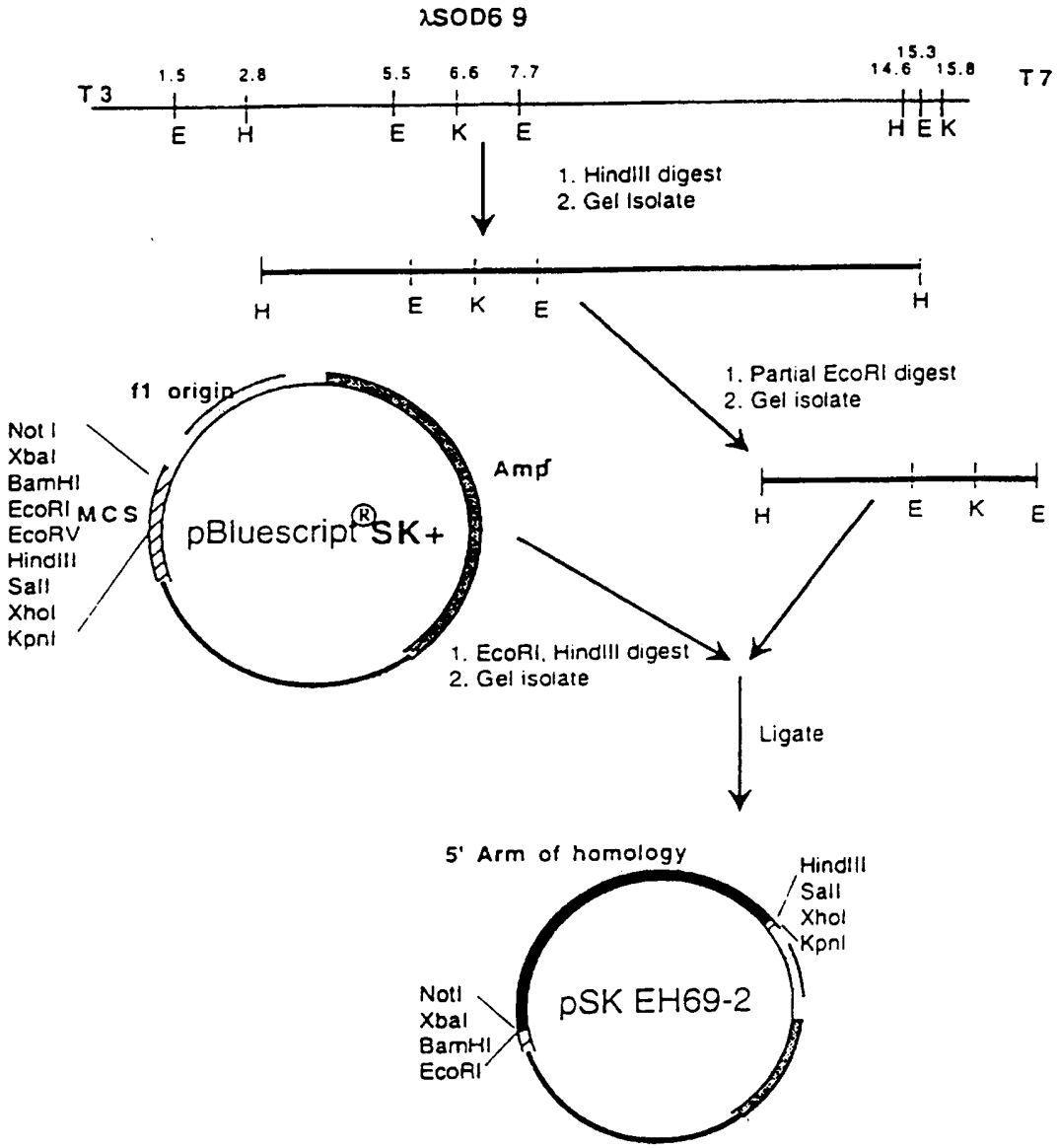

λSOD6 9
T3
T7
1.5
2.8
5.5
6.6
7.7
14.6
15.3
15.8
E H E K E H E K
1. HindIII digest
2. Gel Isolate
H E K E H
1. Partial EcoRI digest
2. Gel isolate
H E K E
f1 origin
Amp
Not I
XbaI
BamHI
EcoRI
MCS
EcoRV
HindIII
SalI
XhoI
KpnI
pBluescript® SK+
1. EcoRI, HindIII digest
2. Gel isolate
Ligate
5' Arm of homology
HindIII
SalI
XhoI
KpnI
NotI
XbaI
BamHI
EcoRI
pSK EH69-2

Fig. 32

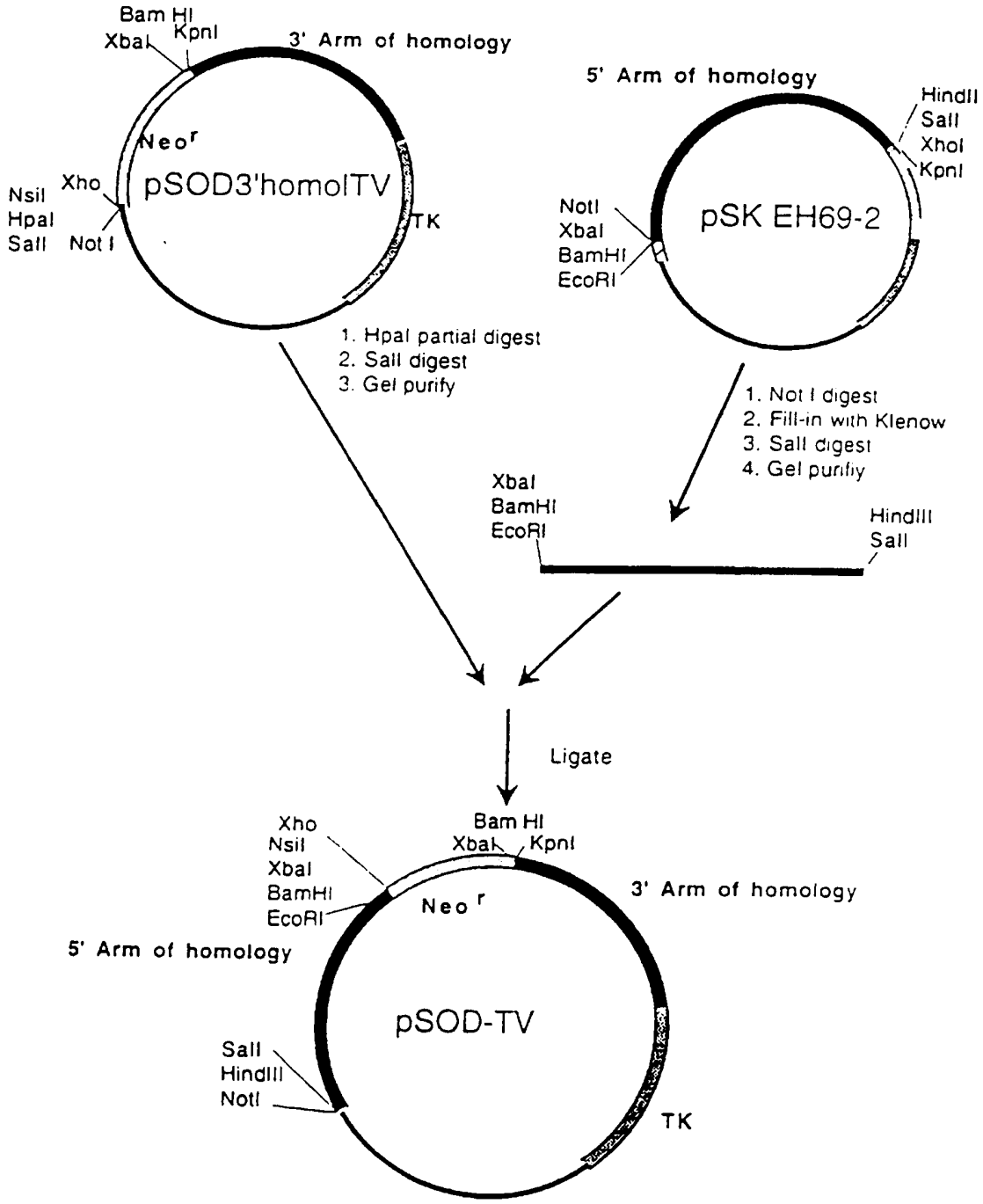

Bam HI
Xbal
KpnI
3' Arm of homology
Neo r
pSOD3'homolTV
Nsil
Hpal
Sall
Xho
Not I
TK
5' Arm of homology
HindII
Sall
Xhol
KpnI
pSK EH69-2
NotI
Xbal
BamHI
EcoRI
1. Hpal partial digest
2. Sall digest
3. Gel purify
1. Not I digest
2. Fill-in with Klenow
3. Sall digest
4. Gel purify
Xbal
BamHI
EcoRI
HindIII
Sall
Ligate
Xho
Nsil
Xbal
BamHI
EcoRI
Bam HI
Xbal
KpnI
3' Arm of homology
Neo r
5' Arm of homology
pSOD-TV
Sall
HindIII
NotI
TK

Fig. 33

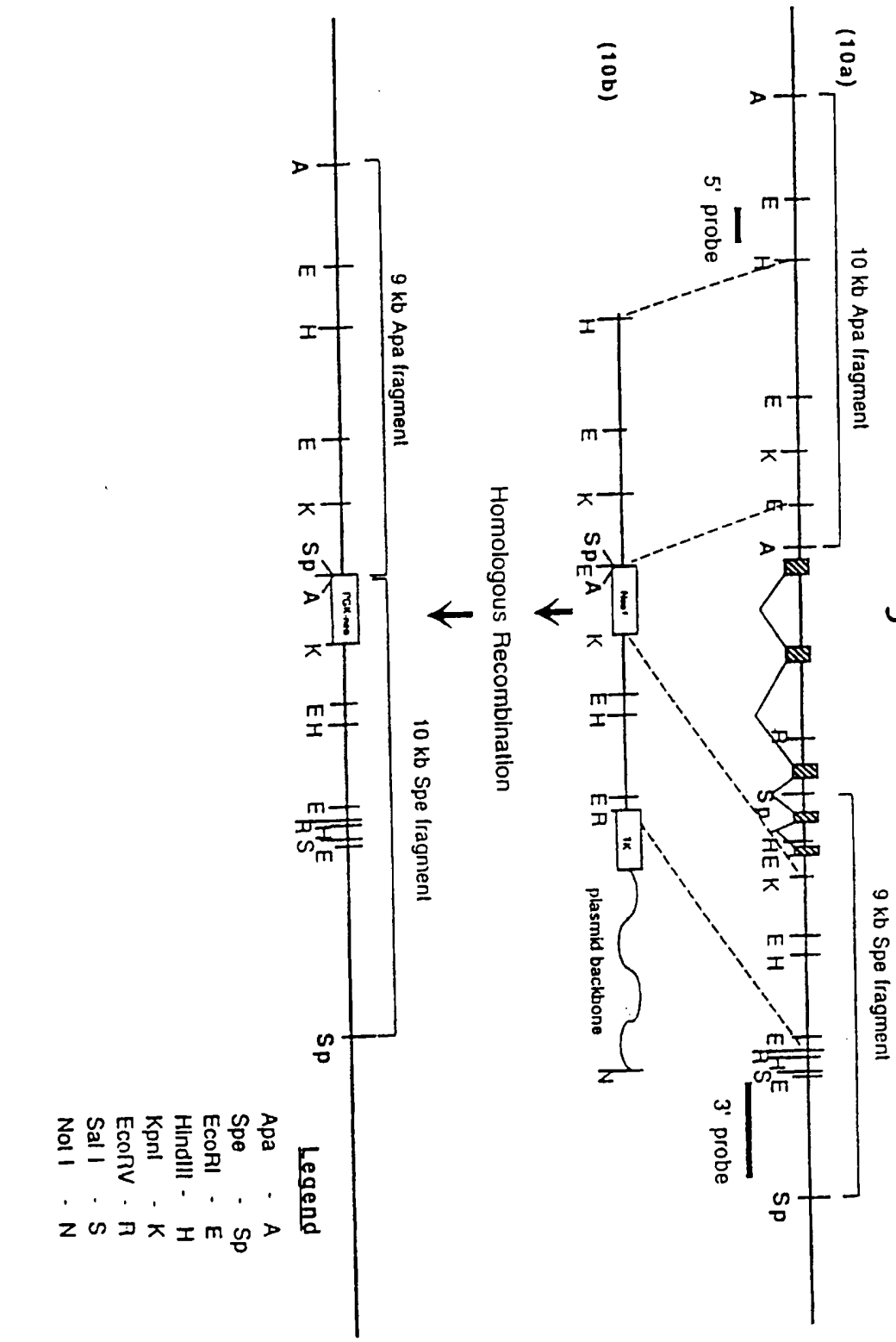
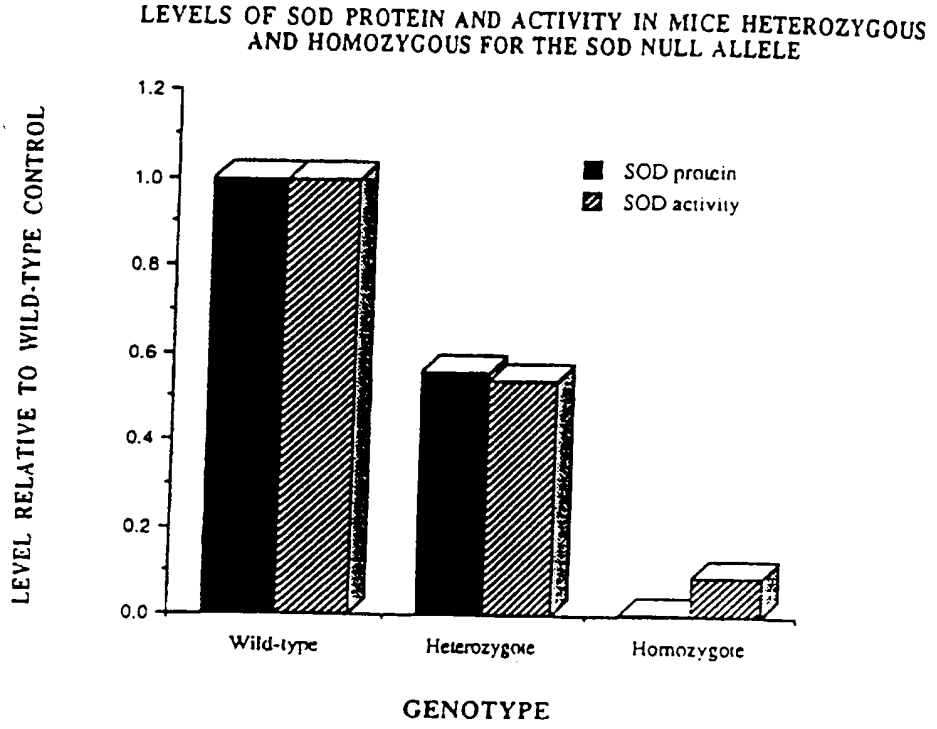

(10a)
(10b)
10 kb Apa fragment
9 kb Spe fragment
5' probe
3' probe
plasmid backbone
Homologous Recombination
9 kb Apa fragment
10 kb Spe fragment
Legend
Apa - A
Spe - Sp
EcoRI - E
HindIII - H
KpnI - K
EcoRV - R
Sal I - S
Not I - N

Fig. 34


LEVELS OF SOD PROTEIN AND ACTIVITY IN MICE HETEROZYGOUS AND HOMOZYGOUS FOR THE SOD NULL ALLELE
LEVEL RELATIVE TO WILD-TYPE CONTROL
1.2
1.0
0.8
0.6
0.4
0.2
0.0
SOD protein
SOD activity
Wild-type
Heterozygote
Homozygote
GENOTYPE

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/05824

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/00 C12N15/12 C12N15/90 C07K14/47 A01K67/027
G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A01K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NEURON, vol. 14, March 1995, CELL PRESS,CAMBRIDGE, MA,USA, pages 661-670, XP000577165 M. CITRON ET AL.: "Generation of amyloid beta protein from its precursor is sequence specific" cited in the application see the whole document --- -/--	1-26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
30 July 1996	09. 08. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hornig, H

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROC. NATL.ACAD SCI., vol. 91, December 1994, NATL. ACAD SCI.,WASHINGTON,DC,US;, pages 11993-11997, XP002009670 M. CITRON ET AL.: "Excessive production of amyloid beta-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation" see the whole document ---	1-26
A	NATURE GENETICS, vol. 1, no. 5, August 1992, NATURE PUBLISHING CO., NEW YORK, US, pages 345-347, XP000577162 M. MULLAN ET AL.: "A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid" cited in the application see the whole document ---	1-26
A	WO,A,93 14200 (TSI CORP) 22 July 1993 see the whole document ---	1-26
A	WO,A,94 12627 (CEPHALON INC) 9 June 1994 see the whole document ---	1-26
P,X	WO,A,95 11968 (ATHENA NEUROSCIENCES INC ;LILLY CO ELI (US)) 4 May 1995 see the whole document ---	1-6,8,20
P,X	EP,A,0 653 154 (HOECHST JAPAN) 17 May 1995 see the whole document ---	1-6,8,20
P,X	WO,A,95 20666 (UNIV MINNESOTA ;HSIAO KAREN (US); BORCHELT DAVID R (US); SISODIA S) 3 August 1995 see page 15, line 13 - line 16; table 2 see page 29, line 21 - page 35, line 10; claims 1-20; figure 2 ---	1-6,8,20
T	NATURE, vol. 380, 14 March 1996, MACMILLAN JOURNALS LTD., LONDON,UK, pages 108-111, XP002009671 J.S. STAMLER: "A radical vascular connection" cited in the application see the whole document ---	1-26

-/--

INTERNATIONAL SEARCH REPORT

In ional Application No
PCT/US 96/05824

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	NATURE, vol. 380, 14 March 1996, MACMILLAN JOURNALS LTD., LONDON,UK, pages 168-171, XP002009672 T. THOMAS ET AL.: "Beta-amyloid-mediated vasoactivity and vascular endothelial damage" cited in the application see the whole document -----	1-26

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...ternational application No.
PCT/US96/05824

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark: Although claims 20, 25, 26 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/05824

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9314200	22-07-93	AU-B-	3336093	03-08-93
		CA-A-	2127450	22-07-93
		EP-A-	0620849	26-10-94
		JP-T-	7506720	27-07-95
WO-A-9412627	09-06-94	NONE		
WO-A-9511968	04-05-95	AU-B-	8079894	22-05-95
		AU-B-	8080994	22-05-95
		CA-A-	2174429	04-05-95
		CA-A-	2174632	04-05-95
		WO-A-	9511994	04-05-95
EP-A-0653154	17-05-95	JP-A-	7132033	23-05-95
		CA-A-	2135595	13-05-95
WO-A-9520666	03-08-95	AU-B-	1909695	15-08-95